

Thesis for the Master's degree in Molecular Biosciences  
Main field of study in Molecular Biology

**Effect of adding 20 nucleotides to the 5' terminus of  
chimeric *rbcL*: GUS transcripts on transcript  
accumulation in chloroplasts of *Chlamydomonas  
reinhardtii***

Anne Witsø

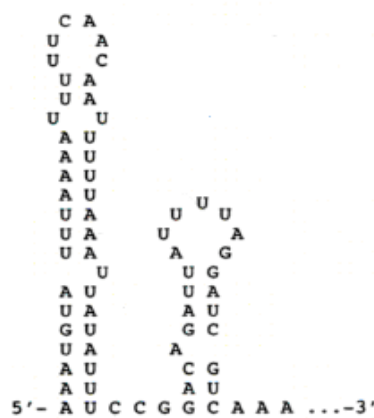
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Faculty of mathematics and natural sciences  
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# **Effect of adding 20 nucleotides to the 5' terminus of chimeric *rbcL*: GUS transcripts on transcript accumulation in chloroplasts of *Chlamydomonas reinhardtii***

By Anne Witsø



## **Abstract**

It has previously been found that the sequence and conformation of a 10-nucleotide element in the 5'untranslated region (5'UTR) of *rbcL* mRNA are important for transcript stability. In this study two variants of a 20-nucleotide sequence were added to the 5'terminus of the *rbcL* 5'UTR in a chimeric [*rbcL* 5'UTR: GUS: *psaB* 3'end] reporter gene construct. The two 5'extensions were predicted to fold into different RNA secondary structures (small and large loops) but did not affect the conformation of the previously identified 10-nucleotide stability element. Addition of the two 20-nucleotide sequences resulted in significantly (~ 99%) reduced accumulation of transcripts of the chimeric reporter gene construct suggesting that the extra nucleotides at the RNA's 5'end rendered the transcripts susceptible to RNase attacks.

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Oslo, December 2006

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## 1 INTRODUCTION

Chloroplasts are typical organelles of photoautotrophic eukaryotic cells which drive a variety of functions, including photosynthesis. According to the endosymbiotic hypothesis of organelle evolution the chloroplast compartment is of eubacterial origin (Bhattacharya and Medlin, 1995; McFadden, 2001). However, the molecular machinery that controls and regulates gene expression in plastids is basically prokaryotic (Hirose et al., 1998; Serino and Maliga, 1998).

In the unicellular green alga *Chlamydomonas reinhardtii*, the plastid genome contains approximately one hundred genes (Maul et al., 2002). Most of these genes encode subunits of either photosynthetic complexes or the chloroplast transcription and translation machinery (Goldschmidt-Clermont, 1998). Many more genes are encoded by the nuclear genome. The nucleus probably dedicates a few thousand genes to chloroplast functions (Rochaix, 2001; Jarvis and Robinson, 2004). In chloroplasts, gene expression is controlled at the transcriptional and post-transcriptional levels (Rochaix, 1996; Goldschmidt-Clermont, 1998). Genetic analysis have revealed a large number of nuclear genes encoding proteins that are synthesized in the cytosol and subsequently are imported in the chloroplast, where they function primarily in post-transcriptional steps of chloroplast gene expression (Rochaix, 1996). This is in contrast to the nuclear genes that are to a much larger extent regulated at the transcriptional level. In chloroplasts, much of the gene expression control has been found to be mediated by differential RNA stabilization events, although significant changes in the transcription rate of some chloroplast genes have been reported during the cell cycle of *Chlamydomonas reinhardtii* (Salvador et al., 1993). The current models of chloroplast RNA stabilization predicts distinct RNA elements (*cis*-acting), which interact with nucleus-encoded (*trans*-acting) factors that protect transcripts from rapid degradation by nucleases in the chloroplasts (Nickelsen, 2003). In general, crucial *cis*-acting determinants for RNA stability in organelle mRNAs have been found in the 5'- untranslated regions (UTRs) of chloroplast transcripts (Salvador et al., 1993; Grunberg-Manago, 1999).

This study looked at how an addition of twenty nucleotides (containing different secondary structures, e.g. stem-loops) to the 5'UTR of the chloroplast encoded *rbcL* gene of *Chlamydomonas reinhardtii* affects the *in vivo* accumulation of transcripts of

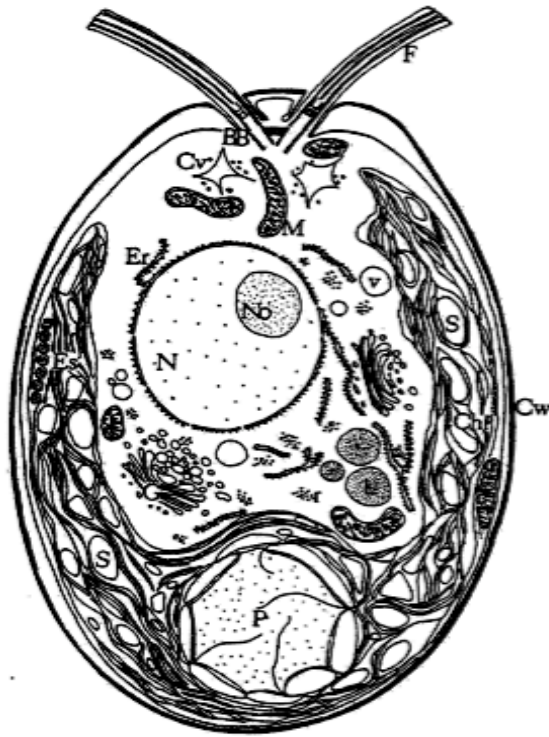
the reporter gene constructs, which consist of the *rbcL* 5'UTR from *Chlamydomonas* fused to the *E.coli uidA* ( $\beta$ -glucuronidase; GUS) gene and the *psaB* 3'-region.

### **1.1 *Chlamydomonas reinhardtii* as a model organism**

Of the large eukaryote unicellular green algae genus *Chlamydomonas*, *Chlamydomonas reinhardtii* (from now on *Chlamydomonas*) is the one most frequently used in the laboratory (Harris, 2001). This strain began to emerge as a laboratory organism, with the work of Ralph A. Lewin and Ruth Sager in the 1940s and 1950s, and it has been the organism of choice ever since. Photosynthetic function is dispensable, provided a reduced carbon source such as acetate is included in the growth medium. The alga can, therefore, be grown under three different regimes: phototrophic growth with CO<sub>2</sub> assimilated through photosynthesis as the unique carbon source, heterotrophic growth in the dark with acetate, and mixotrophic growth in the light with acetate (Harris, 1989). These growth properties have been used extensively to isolate and maintain numerous mutants deficient in photosynthetic activity (Davies and Grossman, 1998). Cell division of this alga can be synchronized by subjecting cells to alternate light and dark cycles (Rochaix, 2001) and their generation time is short, about 5 hours.

*Chlamydomonas* is also quite easy to transform. Transformation of the chloroplast, nuclear, and the mitochondrial genomes have been achieved, allowing extensive research on them (Harris, 2001). The *Chlamydomonas* chloroplast genome can be transformed using microprojectile particle bombardment. The biolistic procedure appears to be the most efficient way of introducing DNA into the chloroplast genome (Boynton et al., 1988).

*Chlamydomonas* is also uniquely suited for research in other areas such as: phototaxis, cell wall synthesis, mating reactions and gametogenesis, and the metabolism of carbon, nitrogen and sulphur (Harris, 2001). It also provides additional insight into eukaryote cells other than yeast, the traditionally used eukaryotic model organism (Rochaix, 1995).



**Fig. 1 Schematic representation of the cell architecture of *Chlamydomonas reinhardtii*.** The wild-type cell averages  $\sim 10 \mu\text{m}$  in diameter. BB, basal bodies; Chl, chloroplast; Cv, contractile vacuole; Cw, cell wall; Er, endoplasmic reticulum; Es, eyespot; F, flagella; G, golgi apparatus; L, lipid body; Mi, Mitochondria; N, nucleus; No, nucleolus; P, pyrenoid; r, ribosomes; S, starch grain; v, vacuole (Harris, 2001).

### 1.1.1 Characteristics of *Chlamydomonas*

Wild-type *Chlamydomonas* cells are oval shaped, average about  $10 \mu\text{m}$  in length and  $3 \mu\text{m}$  in width. They can swim using a breast-stroke beating motion, with the aid of two  $10\text{--}12 \mu\text{m}$  long flagella that are located at the anterior end (Fig.1) (Rochaix, 2001). The cell is enclosed within a cell wall that is built of multiple protein layers without cellulose (Harris, 2001). Cross-sections of cells show prominent morphological compartments, such as the chloroplast, the nucleus and nucleolus (Fig.1). In addition, *Chlamydomonas* possesses another particular organelle, near the cell's equator, an eyespot apparatus, or stigma that senses light. The nuclear membrane is continuous with the endoplasmic reticulum, and one of the Golgi bodies is situated nearby (Fig.1).

Like higher plants, *Chlamydomonas* contains three genetic systems; they are located in the nucleus, the chloroplast, and the mitochondria. The nuclear genome size is estimated at  $\sim 100 \text{ Mb}$  contained on 17 small chromosomes (Harris, 2001; Kathir et al., 2003). The mitochondrial genome is a linear  $15.8 \text{ kb}$  molecule and is considerably smaller than the mitochondrial genomes of plants (Gray and Boer, 1988) which ranges between  $200$  and  $2400 \text{ kb}$ . The chloroplast genome is found to consist of  $200 \text{ kb}$  circular molecules. It is therefore larger than the plastid genomes of land plants, which



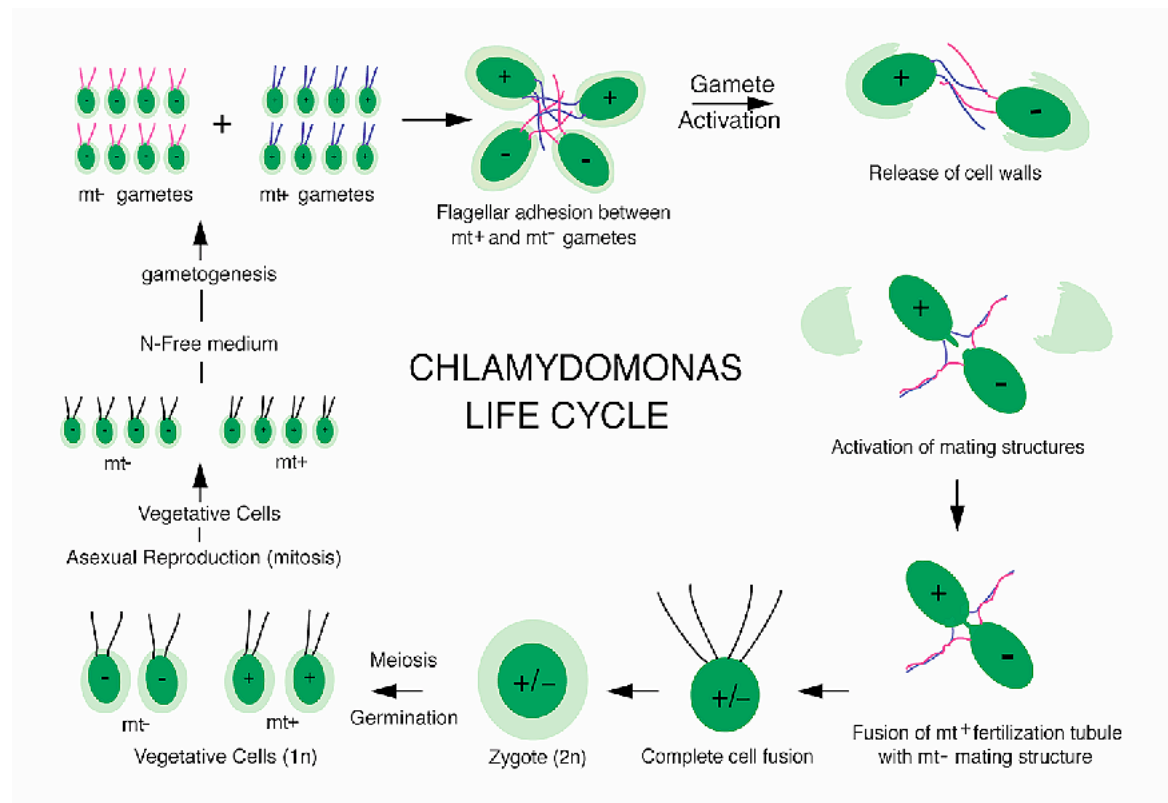
range between 120 and 160 kb (Maul et al., 2002). The informational content of the DNA in plastid genomes is relatively low.

The haploid vegetative cells of *Chlamydomonas*, which are easily cultivated, normally exist as one of two genetically determined species designated mating-type <sup>+</sup> and mating-type <sup>-</sup> determined by alternative alleles of a nuclear gene. In contrast to nuclear genes, which are transmitted to the offspring in a classical Mendelian fashion, chloroplast and mitochondrial genes are normally transmitted uniparentally from the plus and the minus mating-types, respectively (Harris, 1989; Proschold et al., 2005).

### **1.1.2 The *Chlamydomonas* life cycle**

*Chlamydomonas* has a simple life cycle and the stages of the cycle can be evoked in culture (Proschold et al., 2005). Mature haploid cells reproduce asexually and when grown in light-dark cycles, cells divide during the dark phase, usually with two or sometimes three mitotic divisions taking place in rapid succession (Harris, 2001). Gametogenesis is usually induced when vegetative cells are starved for nitrogen (Harris, 2001). When mixed, + and – gametes or mating partners rapidly pair by adhesion of their flagella, fuse, and form a diploid cell that becomes a heavy-walled zygospore. Since an individual gamete can adhere to more than one cell of opposite mating type, clumps of gametes are formed.

Meiosis occurs at zygospore germination, producing four haploid cells in an unordered tetrad; two are of the + and two are of the – mating type (Proschold et al., 2005). Zygospores can remain viable in soil for several years. In the laboratory however, the spores germinate to form motile cells within a few days (Harris, 2001).



**Fig. 2 The life cycle of *Chlamydomonas*.**

The unicellular chlorophyte exhibits sexual as well as asexual reproduction.

### 1.1.3 The chloroplast genome of *Chlamydomonas*

Unlike higher plants which may have as many as 100 chloroplasts per cell, *Chlamydomonas* cells contain a single cup-shaped large chloroplast which occupies the basal two third of the cell (Harris, 2001).

*In vivo*, the genome is organized as a population of monomeric and dimeric circular and linear chromosomes that exists in the range of 50 – 80 molecule copies per chloroplast (Maul et al., 2002). The completion of the chloroplast genome sequence of *Chlamydomonas* was announced 2002 and revealed a 203, 828 bp plastid chromosome (Maul et al., 2002). The circular map of the *C. reinhardtii* chloroplast genome is shown in Fig. 3. The genome is organized in two copies of a 22 kb inverted repeat (IR) sequence (Fig. 3, outer circle), a landmark feature of many plastid genomes. The IRs are separated by two nearly equally sized unique regions of ~ 80 kb. The repeats contain genes for ribosomal RNAs (rRNAs) and the *psbA* operon (Fig. 3). The gene arrangement within the IR is typical of land plants, except that the 23S rRNA is divided by an intron (Rochaix and Malnoe, 1978) and the *psbA* gene is divided by four introns. Whereas introns are present in many chloroplast genes from

plants, there are no other introns in the genome of *Chlamydomonas* besides those that are located in the *psbA*, *psaA* and 23S rRNA genes (Maul et al., 2002).

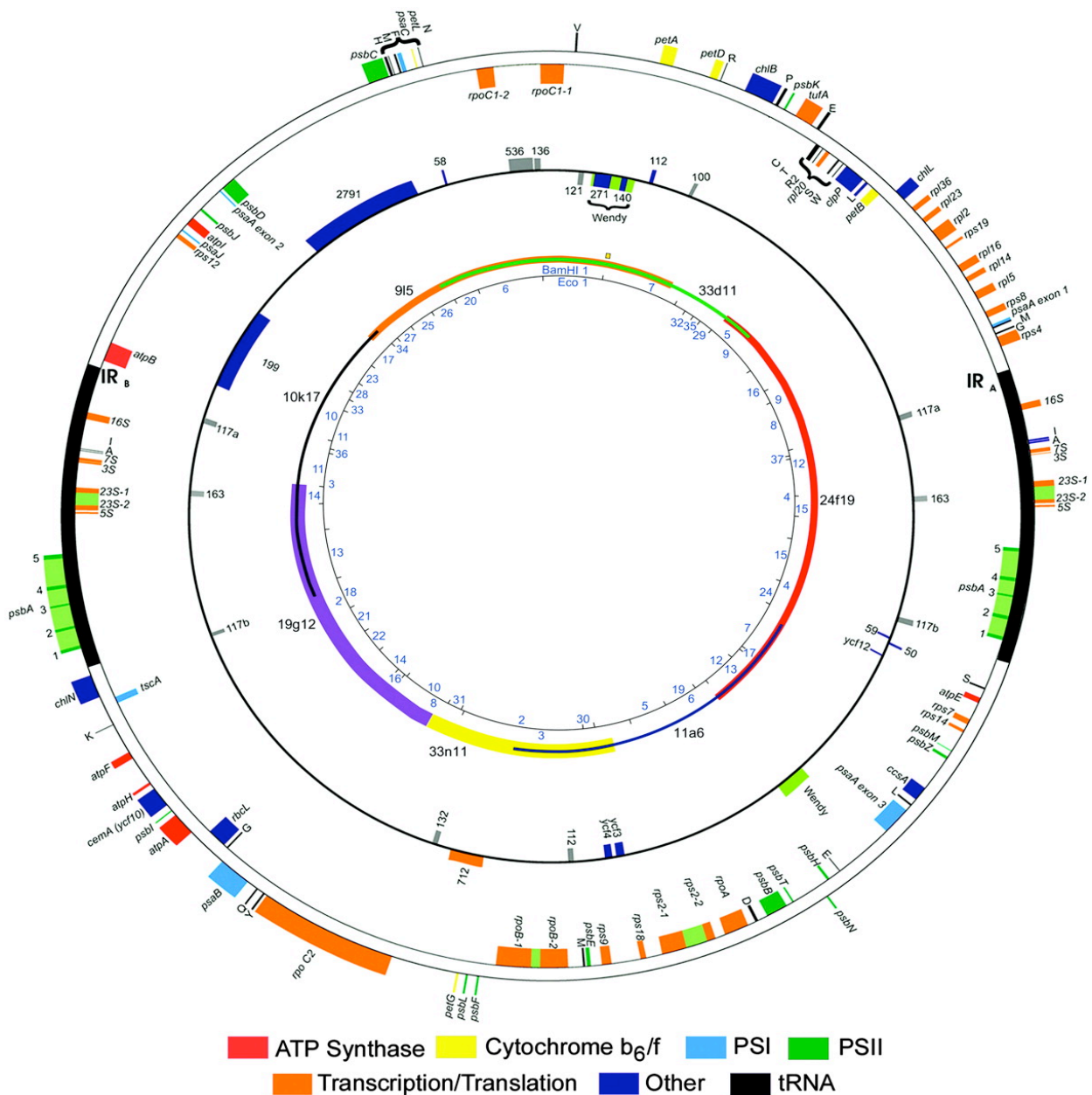
The genome of *Chlamydomonas* has a relatively low number of coding regions (Simpson and Stern, 2002) with a total of 99 expressed sequences (not including gene duplicated in the IR) of which 72 are protein coding genes. Even though the number of open reading frames (ORFs) is smaller than that in most other green algae or land plants (Simpson and Stern, 2002) the ORFs identified in *Chlamydomonas* are clearly specific to the chlorophyte lineage (Maul et al., 2002). Among the protein-coding gene products, 34 genes are related to photosynthetic function. Examples are genes encoding for subunits in photosystem (PS)I and PSII, the cytochrome b6/f-complex, ATP synthase and large subunit of ribulose 1.5-bisphosphate carboxylase (*Fig. 3*). Other protein coding genes are concerned with chlorophyll biosynthesis (ribosomal proteins, large subunit and small subunit) and 31 genes are enrolled in the transcription and translation machinery. Among the latter, an atypical organization of genes encoding RNA polymerase subunits was found in comparison with other photosynthetic organisms (Maul et al., 2002).

Whereas nearly all the structural genes for chloroplast components known in land plants have been identified in *Chlamydomonas* (Harris, 2001) a few ORFs were identified that are unique to *Chlamydomonas* (Maul et al., 2002).

The large size of the chloroplast genome of *Chlamydomonas* is due to the presence of many short dispersed repeats (SDRs), these account for more than 20 % of its sequence (Maul et al., 2002). Moreover, the chloroplast genes are usually larger than their land plant counterparts. While the organization and sequence of these genes on the chloroplast genome in land plants have been conserved (Wakasugi et al., 2001), this conservation does not extend to *Chlamydomonas*. It is probable that the rearrangement of the chloroplast genome is mediated through the SDRs, that may have structural and evolutionary significance. Furthermore, phylogenetic analysis of changes in plastid genome content revealed an accelerated rate of gene loss characterized the *Chlamydomonas/Chlorella* lineage, a phenomenon that might be independent of the proliferation of SDRs (Simpson and Stern, 2002).

A large (65 %) portion of the *Chlamydomonas* genome is transcribed. A general belief has been that *Chlamydomonas* has relatively few polycistronic gene clusters (Barkan and Goldschmidt-Clermont, 2000), but recent findings reveal that 40

% of the genes might be dicistrons or polycistrons. Genes in vascular plant plastid genomes are mostly organized into polycistronic operons (Drapier et al., 1998).



## 1.2 Chloroplast gene expression

Plastids, like mitochondria, are considered to be genetically semiautonomous. The photosynthetic complexes as well as many other proteins in the chloroplast are composed of subunits encoded by both the nuclear and the chloroplast genomes, which necessitates coordinated expression of genes from the two compartments. Several nuclear mutants defective in chloroplast gene expression appear primarily to affect post-transcriptional steps of gene expression including RNA processing and stability (Barkan and Goldschmidt-Clermont, 2000; Herrin and Nickelsen, 2004; Stern et al., 2004). The genes disrupted by the mutations are thought to encode numerous nucleus-encoded factors that mediate the crosstalk between the chloroplast and nuclear genome. The molecular machinery that controls and regulates gene expression in chloroplasts is basically prokaryotic. Reflecting the bacterial ancestry of plastids are, for instance, the structures of plastid RNA polymerases (Maliga, 1998), the sequences of plastid gene promoters (Igloi and Kössel, 1992), prokaryotic ribosome binding sites (Hirose et al., 1998) and structures of other elements, like stem loops at the 3' ends of plastid transcripts (Rott et al., 1998).

### 1.2.1 Chloroplast transcription

#### 1.2.1.1 Promoters

Typical eubacterial-like promoters have been identified in the plastid chromosome of *Chlamydomonas* (Klein et al., 1992; Klinkert et al., 2005). The first type resembles the *E. coli* sigma 70-type, which contains the consensus sequence elements TTGACA and TATAAT at positions -35 and -10, respectively and a promoter that lacks the -35 element but has an extended -10 (TATAATAT) sequence. The latter seems to be the common promoter of protein coding genes in *Chlamydomonas* chloroplasts, whereas the sigma 70-like promoter is found in front of the ribosomal RNA genes. The basic promoter (TATAATAT) of the *rbcL* gene in *Chlamydomonas* is an example of a promoter with an extended -10 sequence and can be sufficient to direct transcription in the chloroplast (Salvador et al., 2004).

Other genes that contain only the -10 element have been found upstream of the *atpB*, *psbD* or *psbA* genes for instance. So far, this element has so far been shown to be sufficient for promoting transcription in the case of *atpB* (Blowers et al., 1990; Klein

et al., 1992). Furthermore, elements enhancing transcription were identified within inter- and intragenic regions of chloroplast genes (Klein et al., 1994; Anthonisen et al., 2002).

Chloroplast promoters of higher plants can be grouped into at least two classes: first, the bacterial sigma 70-type promoters containing -10 and -35 consensus sequences and second, promoters with a loose consensus sequence near the transcription initiation site (Igloi and Kössel, 1992; Silhavy and Maliga, 1998). Variants of the latter type of promoter have been found (Sriraman et al., 1998).

#### **1.2.1.2 RNA Polymerases**

Higher plant plastid genomes are transcribed by two different RNA polymerases (RNAPs): the phage-like nuclear-encoded NEP (single-subunit RNAP) and the plastid-encoded eubacterial-like PEP (multi-subunit RNAP) (Hess and Börner, 1999; Shirano et al., 2000). The NEP has been found to transcribe housekeeping genes whereas the transcription of photosynthesis-related chloroplast genes is primarily accomplished by PEP. Furthermore, the development of photosynthetically active chloroplasts requires both PEP and NEP.

The catalytic core of PEP consists of  $\alpha$ ,  $\beta$ ,  $\beta'$  and  $\beta''$  subunits encoded by the plastid genes *rpoA*, *rpoB*, *rpoC1* and *rpoC2*. PEP is also thought to require a nucleus-encoded sigma<sup>70</sup>-like factor (SLF), this specifies transcription initiation downstream of the promoter consensus sequences which resemble bacterial promoters of the -10/-35 sigma 70 type. The nucleus-encoded NEP transcribes genes from the other type of promoter (Maliga, 1998; Hess and Börner, 1999). A large number of plant genes encoding SLFs have been identified (Lahiri et al., 1999; Tan and Troxler, 1999; Shirano et al., 2000). The SLFs of land plants show developmental and tissue-specific regulation which may be a possible basis for the requirement of several sigma-like transcription factors to fine-tune plastid gene expression (Tozawa et al., 1998; Lahiri and Allison, 2000).

It has been suggested that *Chlamydomonas* chloroplasts, unlike those of higher plants, lack NEP-like enzymes, since attempts to disrupt PEP-encoding genes have failed (Goldschmidt-Clermont, 1991; Rochaix, 1995; Fischer et al., 1996), and because virtually all its transcription is sensitive to rifampicin, an inhibitor of PEP but not NEP (Eberhard et al., 2002). In addition, in *Chlamydomonas*, *rpo* genes encoding

a PEP are present in the chloroplast genome, but a NEP homologue has not yet been detected in *Chlamydomonas* (Lilly et al., 2002). Thus, genes in the *Chlamydomonas* chloroplast appear to be transcribed exclusively by the PEP protein complex, which thus recognizes at least two types of promoter, the sigma 70-type and a promoter with an extended -10 region.

Interestingly, in contrast to the abundance of up to several (6 ) SLFs in plants, there is only one sigma factor in *Chlamydomonas* (Bohne et al., 2006). Conserved sequences between sigma factors have been cloned and characterized biochemically (CrRpoD). Based on *in vitro* studies it has been suggested that CrRpoD, a protein highly similar to the *E.coli* sigma factor, functions as the sole PEP specificity factor in the chloroplast of *Chlamydomonas*. In conclusion, CrRpoD may regulate all plastid transcription in *Chlamydomonas* (Alexandra et al., 2006).

### **1.2.2 Post-transcriptional processing**

RNA processing is a general term to describe the modifications of a newly synthesized RNA molecule. Primary chloroplast transcripts (pre-mRNAs) in the chloroplasts of plant and algae are processed in a number of ways, including intron splicing, editing, internal cleavage of polycistronic RNAs, and endonucleolytic or exonucleolytic cleavages at the transcripts'5'and 3'ends. These events are regulated to a great extent by nucleus-encoded chloroplast RNA-binding proteins (cpRBPs). These have distinct functions, such as exo-or endoribonucleases, suggesting potential differential regulation of chloroplast gene expression. Results of this processing affects the stability, translatability and degradation of mature mRNA.

#### **1.2.2.1 Intron splicing**

Splicing is an important posttranscriptional step in chloroplast gene expression, since many chloroplast plant genes including rRNA, tRNA, and mRNA-genes are interrupted by introns that must be spliced. In *Chlamydomonas* however, only three chloroplast genes contain introns (section 1.1.3). Splicing occurs either in the familiar *cis*-pathway, connecting different exons in the same transcript, or in the *trans*-pathway, joining exons in different transcripts.

Most chloroplast introns fall into three major classes, group I, II and III. Members of each class possess a characteristic secondary structure that mediate

different ways of splicing. Some introns of group I and II exhibit self-splicing *in vitro*, though it is believed that *in vivo* splicing reactions require *trans*-factors, including ribonucleases (Rochaix, 1996).

As for regulation of transcript stability there is also evidence of regulation of splicing by developmental and environmental cues. For example in *Chlamydomonas*, splicing of the intron-containing *psbA* pre-mRNA is accelerated in light. Mutants of one of the *psbA*-intron sequences in this gene provided evidence that *psbA* splicing is under redox-control and that efficient splicing is important for photosynthetic growth of this organism (Deshpande et al., 1997; Lee and Herrin, 2003).

Besides *cis*-splicing there is *trans*-splicing in *Chlamydomonas* during the maturation of the *psaA* message (Turmel et al., 1995).

#### **1.2.2.2 Intercistronic processing**

Several plastid genes are organized mainly as operons. Most of these genes are transcribed into polycistronic precursors that may be later cleaved into monocistronic transcripts. This processing is part of the machinery that renders the transcripts competent for translation and is therefore important in chloroplast gene expression (Eibl et al., 1999; Barkan and Goldschmidt-Clermont, 2000). It might be asked whether this processing has a functional significance, since in bacteria all proteins can be translated from a primary polycistronic transcript. Examples of the importance of intercistronic processing however, comes from several plants. The tobacco *in vitro* translation system has been used to analyze the importance of processing within the *psaC-ndhD* intergenic region (Barkan A, 1998) and mutants with defects in chloroplast intercistronic processing leading to translational blocks have been identified in maize and *Arabidopsis* (Barkan and Goldschmidt-Clermont, 2000).

Ribosomal RNAs are also transcribed as precursors that must be processed in order for ribosome assembly to occur, for e.g the 23S rRNA in *Chlamydomonas* (Holloway and Herrin, 1998). The enzymes involved in intercistronic mRNA processing, are thought to include both endo- and exonucleases and might be the same enzymes involved in the maturation of the 3'UTR and 5'UTR (section 1.2.2.4).

#### **1.2.2.3 RNA editing**

Another common form of RNA processing, particularly in organelles, is RNA editing. While it is known that plant chloroplasts modify transcripts by RNA editing (Maier et



al., 1996), there is no evidence for editing in *Chlamydomonas* chloroplasts (Maul et al., 2002). RNA editing in chloroplasts usually results in C to U conversions that generally create start codons, or reintroduce a conserved amino acid into the protein (Maier et al., 1996).

As a reliable *in vitro* editing system has not been reported for chloroplasts, analyses of *cis*-sequences required for editing have been performed *in vivo* using chloroplast transformation (Bock et al., 1996; Reed et al., 1997). No consensus in sequence or secondary structure is known for *cis*-acting elements and none of the *trans*-acting factors involved in the above mentioned conversion have been identified.

#### **1.2.2.4 5' - and 3' maturation**

Apart from the internal regions of chloroplast RNAs, the 5'UTRs and 3'UTRs have attracted special attention with regard to their potential role in regulation of post-transcriptional gene expression steps. Most chloroplast transcripts are subjected to nucleolytic processing at the 3' terminus (3'UTR), because transcription typically continues beyond the 3'UTR of the gene (Rott et al., 1996). Inverted repeats (IR) occur downstream of many chloroplast genes, and play an important role in determining the 3'end of these mRNAs (Rott et al., 1998).

IRs can often form stem-loop structures, which stabilize the transcripts. Removal of the stem-loop structure, for instance, by internal endonucleolytic cuts results in rapid degradation of the RNAs.

The stem-loop structure often serves as processing signal for correct 3'end formation in both plants and algae (Rott et al., 1996). This 3'end formation involves a two-step process, an initial endonucleolytic cut downstream of the stem-loop structure and a subsequent exonucleolytic trimming in the 3'→5'direction.

However, it seems that the endonucleolytic cleavage site is not always necessary for 3'end formation (Rott et al., 1999) and alternative pathways may exist.

Many transcripts are also processed at the 5'end, although this has not been thoroughly studied. Two types of processing are known-endoribonuclease cleavage and 5' to 3'- exonuclease trimming. Ribonucleolytic cleavage is often observed by finding two transcript populations for the same gene, one with a 5'end corresponding to a processing site. In *Chlamydomonas*, processed transcripts were found to be the only translatable transcript form (Nickelsen et al., 1999; Nickelsen et al., 1999; Vaistij et al., 2000).

### 1.2.3 mRNA stability

RNA longevity is of major importance for chloroplast gene expression. Transcription rate and transcript stability are both involved in determining availability of transcripts for the translation machinery. In many cases an increased abundance of mRNAs cannot be explained by changes in relative transcription rates (Wang et al., 2002; Hambræus et al., 2003). Differential changes in mRNA levels can often not be accounted to transcription fluctuations (Mayfield et al., 1995), but to changes in RNA stability that regulate RNA levels in response to developmental and environmental factors.

mRNA stability is often described in terms of half-lives. The mRNA decay rate is a major determinant of mRNA abundance and it has been found that the longevity of an individual mRNA population can fluctuate many-fold following a change in mRNA half-life. It is generally assumed that the average stability of transcripts in different species is linked to the cell cycle period of that species, i.e. the shorter the cell cycle the shorter the half-lives of transcripts. This could allow the organism to adjust the composition of their RNA pool (transcriptome) when environmental (i.e. light) or internal conditions change. In *Chlamydomonas*, chloroplast transcripts have been found to be generally long-lived (half-lives varying from a few to several hours) (Salvador et al., 1993) in comparison to *E.coli* transcripts that have a half-life of a few minutes (Bouvet and Belasco, 1992).

In chloroplasts the stability of individual mature mRNAs depends on multiple factors such as external regulative (e.g. light/dark cycles) and internal regulative (*cis*-acting sequences and *trans*-acting proteins).

#### 1.2.3.1 External factors

Light is the most important external signal that influences chloroplast gene expression. Light/dark cycles can adjust the composition of the RNA pool and may cause differences in both stability and translatability. For example when grown in 12–hours dark/12–hour light cycles the half-life of the endogenous *rbcL* transcripts in *Chlamydomonas* chloroplasts is 21 hours in the dark, and only 3.5 to 5 hours in light. Furthermore, a general increase in transcription has been shown in the light period

and enhanced stability in the dark (the latter in response to a low transcription rate) (Nickelsen, 1998).

The effect of light on the stability of chloroplast transcripts has been studied in some detail in *Chlamydomonas* (Salvador et al., 1993, 1993). For all chimeric transcripts tested, made up of a reporter gene construct containing the *rbcL* promoter and the 5'UTR fused to the *uidA* reporter gene, the stability (half-life) was significantly higher in the dark than in the light when cells were grown in light-dark cycles. In contrast to this, it was found that endogenous *rbcL* gene transcripts are relatively stable both in dark and in light. Although *rbcL* transcript stability decreased somewhat in the light, this was compensated by an increased transcription rate, keeping transcript levels at steady state (Salvador et al., 1993). Also in plant cells (tobacco) the relative levels of *uidA* mRNA decreased when cells were shifted from darkness into light. Experiments in which photosynthetic electron transport was inhibited suggested that the RNA degradation system-similar to the transcriptional and translational machinery - could be influenced by the redox potential of the chloroplast stroma (Pfannschmidt and Allen, 1999; Salvador and Klein, 1999; Bruick and Mayfield, 1999).

### **1.2.3.2 Cis-acting elements**

Due to their stabilizing or destabilizing nature, *cis*-acting sequences in the mature RNA of chloroplast-encoded genes are important for determining longevity.

In eukaryotes, elements important for mRNA longevity have been delineated primarily in the 3'UTR of transcripts (Ross, 1996; Gutierrez et al., 1999; Mitchell and Tollervey, 2001). In bacterial and organelle mRNAs (prokaryote-type mRNAs), essential determinants of mRNA longevity seem to be located mostly in the 5'UTR (Salvador et al., 1993; Grunberg-Manago, 1999).

In chloroplasts the 3'-UTR appears to affect mRNA stability and 3'end maturation, but does not seem to have any influence on translation efficiency (Stern and Gruissem, 1987; Drager et al., 1996). Most chloroplast 3'UTRs contain an inverted repeat (IR) that can fold to form a stem-loop structure. The 3'UTRs have been shown to function in mRNA stabilization in *Chlamydomonas* (Blowers et al., 1993; Rott et al., 1998). However, studies on the 3'UTR of the *Chlamydomonas rbcL* gene have revealed that the 3'end probably is not important in determining the stability of the

RNA, but plays a role in end maturation (Blowers et al., 1993). Examples of *cis* – acting elements are also found in endogenous coding regions (Singh et al., 2001). Mutations in these regions reduce the longevity of transcripts and disrupt essential RNA-RNA interactions.

In contrast to the 3'UTRs, the 5'UTRs appear to be of crucial importance for both mRNA stability and translation efficiency, as indicated by various analyses of chimeric transcripts and nuclear mutants (Rott et al., 1996; Higgs et al., 1999; Vaistij et al., 2000). Sequences in the 5'UTR are determinants of transcript longevity in mRNA of *Chlamydomonas* chloroplast genes. *Cis*-acting expression determinants have been found in *psbD*, *petD*, *rbcL* and *psbB* transcripts (Higgs et al., 1999; Nickelsen et al., 1999; Anthonisen et al., 2001; Singh et al., 2001). The first indications for the role of chloroplast RNA 5' regions were obtained for the *rbcL* gene (Salvador et al., 1993). Precise mapping of RNA stability elements within the *rbcL* 5'UTR revealed a 10-nt *cis*-acting sequence required for general stability and thus RNA accumulation *in vivo* (Anthonisen et al., 2001). In higher plants, *cis*-sequences in 5'UTRs of transcripts are crucial for stabilization of the *rbcL* mRNA (Shiina et al., 1998).

Some of these *cis*-elements contain sequences that have the potential to form secondary structures (Drager et al., 1996; Higgs et al., 1999; Anthonisen et al., 2001). For instance, the previously identified *cis*-element in *rbcL* 5'UTR, which was shown to be crucial for stability, is partly included in the predicted stem-loop structures of the *rbcL* 5'UTR. It is thought that the stem-loop at the 5' terminus has a formative function in that it mediates folding of the 10-nt sequence around its base into a specific RNA conformation. It is assumed that disrupting this conformation renders transcripts completely unstable (Anthonisen et al., 2001).

It is further believed that secondary structures (e.g. stem-loops) formed in the 5' region of chloroplast transcripts are common *cis*-acting RNA stabilizing elements (Higgs et al., 1999; Fargo et al., 2000) and might be essential in protecting against nucleolytic degradation. Their exact role in preventing degradation is uncertain, but it is assumed that these are binding sites for *trans*-acting factors (Memon et al., 1996; Higgs et al., 1999; Zou et al., 2003). In the case of the *rbcL* 5' terminal stem-loop, it is not the structure that shields transcript from attack or degradation by ribonucleases, but the short single stranded region between the stem-loop structures that is thought to bind *trans*-acting factors (Suay et al., 2005).

Finally, it is possible that different chloroplast mRNA species contain different sequences and structures at their 5' ends that are involved in stabilizing transcripts. This is due to the apparent lack of sequence and a structural consensus among 5' sequences of chloroplast mRNAs in general (Anthonisen et al., 2001).

### **1.2.3.3 *Trans-acting factors***

Findings suggests that interactions of regulatory proteins (*trans-acting factors*) with the *cis-acting* sequence elements in the 5' UTR of mRNAs are involved in light/dark regulation of transcript longevity in *Chlamydomonas* chloroplasts (Salvador and Klein, 1999). Light-regulated binding of proteins to the mRNAs may be regulated, at least in part, by changes in redox-state generated by photosynthesis (Kim and Mayfield 1997). In addition to light, i.e redox processes, phosphorylation appears to play an important role in regulating chloroplast mRNA binding proteins (cpRBP) activities in chloroplasts (Liere and Link, 1997). Redox carriers in the chloroplast are thought to be potential transmitters of light stimuli to the stabilization apparatus (Salvador and Klein, 1999).

In stabilization of chloroplast mRNA, *trans-acting factors* interact with *cis-acting* elements. These proteins are thought to stabilize transcripts by reducing ribonuclease access and promote RNA maturation processes. RNA binding proteins (cpRBPs) are abundant in the chloroplast stroma and are found to be associated with ribosome-free mRNAs (Nakamura et al., 1999, 2001).

Several chloroplast RNA-binding proteins have been detected by UV-crosslinking and gel-shift assays (Nakamura et al., 2001). These have been found to bind to the 5' or 3' UTR of mRNAs. Most cpRBPs seem to be organized in supramolecular multi-protein complexes (Rochaix, 1996; Nickelsen, 2003; Zerges et al., 2003).

Over the past 10 years, a number of cpRBPs have been identified and characterized both biochemically and by analyzing photosynthetic mutants. Most of these nuclear mutations encode regulatory factors affecting post-transcriptional steps, including RNA processing and stability, whether isolated in higher plants (Barkan and Goldschmidt-Clermont, 2000; Stern et al., 2004) or in *Chlamydomonas* (Herrin and Nickelsen, 2004). Interestingly, these mutations seem to act in a gene specific manner, with one nuclear mutation affecting the stability of one

or a few chloroplast transcripts, despite having wild-type levels of transcription. This might be a difference between *Chlamydomonas* and higher plants, where related mutations more often seem to influence the stability of a greater number of transcripts (Meurer et al., 1996).

Most strikingly, however, the function of most nucleus-encoded regulatory RNA stability factors determining transcript half-lives from *Chlamydomonas*, is mediated - directly or indirectly - via the 5'UTRs of the mRNA they regulate (Nickelsen, 2003). These mRNAs include those of the *psbD*, *petD* and *psbB* genes, which are unstable in the respective nuclear mutants *mbd1*, *mcd1* and *mbb1* (Nickelsen et al., 1994; Drager et al., 1998; Vaistij et al., 2000). In these three mutants RNA accumulation is completely abolished, as is photoautotrophic growth. Furthermore, since the 5'UTRs are generally the sites of translation regulation, it is difficult to know which of the two processes, stability or translation, might be influenced by the RNA binding protein.

Finally, IRs found in the 3' UTR associated with RNA stability, may also interact with specific nuclear *trans*-acting factors (Levy et al., 1999; Meierhoff et al., 2003).

#### **1.2.4 mRNA degradation**

The molecular mechanisms of RNA degradation in chloroplasts have been studied in some detail and could resemble certain aspects of RNA degradation in *E.coli* (Carpousis et al., 1999; Hayes et al., 1999; Schuster et al., 1999; Steege, 2000). For a typical chloroplast mRNA, with a 3'IR, RNA degradation is initiated by endonucleolytic cleavage of the RNA molecule which removes the 3' stem-loop structures. This is followed by addition of a destabilizing poly (A)-tail to the 3' end of the resulting fragments that makes them susceptible to nucleases. In vascular plants and cyanobacteria polynucleotide phosphorylase (PNPase) acts both as a poly (A)-polymerase that adds multiple A residues to the 3' ends of chloroplast RNAs and a 3' → 5' exonuclease that subsequently removes the polyadenylated cleavage products (Kudla et al., 1996; Lisitsky et al., 1996; Hayes et al., 1999). Also in *Chlamydomonas*, polyadenylation has been detected at mature 3' ends in major classes of RNAs i.e., mRNAs, tRNAs and rRNAs (Komine et al., 2000).

In addition, a poly (A)-independent degradation pathway is proposed to exist in

chloroplasts. This pathway is catalyzed by exonucleases RNase II and PNPase in 3'→5' direction (Bollenbach et al., 2004).

In *E.coli*, the endonuclease RNaseE, which is part of the *E.coli* degradosome (Carpousis et al., 1999), initiates degradation. Several endonucleases have been reported in chloroplasts, but none of them resembles the bacterial RNaseE (Nickelsen and Link, 1993; Yang et al., 1996; Monde et al., 2000), yet some nuclear plant genomes encode RNase-E-like proteins (Slomovic et al., 2006) and homologues of the *rne* gene encoding RNase E have been found in algal plastid genomes. Notably, no RNase E-homologue has been identified in the *Chlamydomonas* genome to date. Among the chloroplast endonucleases characterized in more detail are the p54 protein from mustard and the CSP41 protein from spinach plastids (Nickelsen and Link, 1993; Yang et al., 1996). The activities of both enzymes are controlled by their redox and phosphorylation states (Yang et al., 1996).

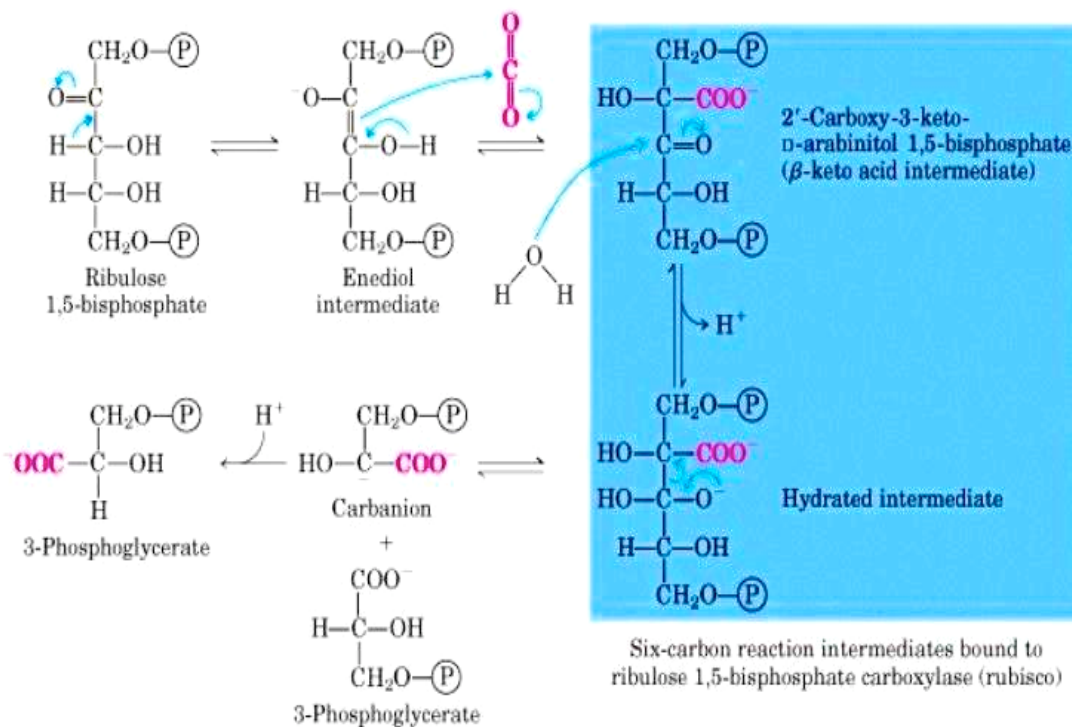
Relatively little is known about the degradation machinery that attacks the transcripts' 5' regions, but in *Chlamydomonas* there is evidence suggesting the presence of 5'→3' exonucleolytic activities for the chloroplast *petD*, *psbD* and *psbB* genes (Drager et al., 1998; Drager et al., 1999; Nickelsen et al., 1999; Vaistij et al., 2000). Evidence comes from inserting polyguanosine (pG)-tracts into the 5'UTRs of these chloroplast mRNAs. Similar to the 3'UTRs, these tracts were shown to have a protective function, since pG-tracts form a complex tertiary structure that known exonucleases cannot progress. Poly- G-tracts have also been shown to impede the activity of 5'→3' exonucleases in yeast (Decker and Parker, 1993). Interestingly, 5'→3' exonuclease activity found in chloroplasts have no known counterparts in bacteria (Monde et al., 2000).

### **1.3 RuBisCo**

In this study, a focus has been placed on the chloroplast *rbcL* gene encoding the large subunit of the ribulose-1.5-bisphosphate carboxylase-oxygenase (RuBisCo) - protein complex. Rubisco is a key-enzyme in the Calvin cycle, a set of reactions also called carbon fixation and normally takes place in the stroma of chloroplasts in photosynthetic organisms. In *Chlamydomonas* and most algae, the pyrenoids, distinctive bodies within the chloroplast (Harris, 2001) are composed primarily of Rubisco. The Rubisco holoenzym of *Chlamydomonas* is composed of eight 55-kDA large subunits (LSU) encoded by the chloroplast *rbcL* gene and eight 16-kDA small

subunits encoded by a family of two nearly identical *rbcS* genes in the nucleus (Spreitzer and Salvucci, 2002; Spreitzer et al., 2005).

The large subunit contains the enzyme's catalytic site while the function of the small subunit is unknown (Voet and Voet, 2004). Rubisco catalyzes either the carboxylation or oxygenation of ribulose-1,5 biphosphate (also known as RuBP) with carbon dioxide or oxygen. The proposed mechanism of the enzyme can be seen below (Fig.4).



**Fig. 4 Probable reaction mechanism of the carboxylation reaction catalyzed by RuBP Carboxylase.** Ribulose biphosphatase catalyzes the conversion of ribulose-1, 5-biphosphate (RuBP) plus CO<sub>2</sub> into two molecules of 3-phosphoglycerate (3PG). The first intermediate in the reaction is an enediolate that forms by abstraction of the C3 proton. This is the rate-determining step. The enediolate nucleophilically attacks the CO<sub>2</sub> to yield the second intermediate, a β-keto acid. The β-keto acid is attacked by water to yield a third intermediate that rapidly breaks down into two molecules of 3-phosphoglycerate (3PG) (Voet and Voet, 2004).

Source: [www.cm.utexas.edu/academic/courses/Spring2002/CH339K/Robertus/overheads-3/ch19\\_rubisco.jpg](http://www.cm.utexas.edu/academic/courses/Spring2002/CH339K/Robertus/overheads-3/ch19_rubisco.jpg).

#### 1.4 Scope of the study

The chloroplast gene *rbcL* encodes the large subunit of the CO<sub>2</sub>-fixing enzyme ribulose-bisphosphate carboxylase, Rubisco (section 1.3).

This study is part of a bigger project, in which important nucleotides in the *rbcL* 5'UTR in the chloroplast of *Chlamydomonas* are being mapped. Transcripts of the *rbcL* 5'UTR are predicted to fold at their 5' end into two stem-loop structures,



separated by a single stranded region that is part of a previously identified *cis*-acting element required for stability (Anthonisen et al., 2001).

A previous study showed that adding 6 unpaired nucleotides to the RNA's 5' terminus did not destabilize transcripts of the *rbcL* 5'UTR (Suay et al., 2005). In contrast, a recent study found that adding 10 unpaired nucleotides to the 5' terminus of the *rbcL* 5'UTR completely destabilized the transcripts (Kristina Zarins, personal communication).

The aim of this project was to investigate in more detail the factors important for stability of transcripts containing an extended 5' terminus. Two variants of a 20 nucleotides sequence were added to the 5' termini of the transcripts' *rbcL* 5'UTR. These sequences did not alter the original secondary structure, but were predicted to form different small and large stem-loops at the 5' ends.

## 2 MATERIALS AND METHODS

### 2.1 Strains and Culture conditions

#### 2.1.1 *Escherichia coli*

All cloning steps were carried out in the recombination deficient *E.coli* strain TB1. Bacteria were grown at 37°C for 12-16 hours in *Luria-Bertania* (LB)- liquid medium while shaking/rotating or on solid agar LB-plates, both containing ampicillin (60 µg/ml).

##### 2.1.1.1 Competent cells

For preparation of competent cells, CaCl<sub>2</sub>-treatment was performed (Sambrook and Russel, 2001).

##### 2.1.1.2 Transformation of competent cells

Competent cells were transformed by heat-shock treatment essentially as described (Sambrook and Russel, 2001). After transformation, cells were plated on ampicillin-LB (LBA) - agar plates for selection.

##### 2.1.1.3 Isolation of plasmid DNA

Colonies of selected transformants were picked and grown overnight on a rotator in LB medium containing ampicillin (60 µg/ml).

For control digestions of plasmids, small-scale plasmid isolation from *E.coli* were carried out according to the mini-prep protocol (Sambrook and Russel, 2001). For minipreps, single colonies each representing the progeny of a single transformed bacterium, were picked and grown to stationary phase in 3 ml LB medium.

DNA from minipreps, still contains RNA. DNA for cloning and transformation of *Chlamydomonas* was isolated by a large-scale method, maxiprep<sup>1</sup>, as described (Sambrook and Russel, 2001).

Long-term storage of *E.coli* cells was done in a 15% glycerol at - 80°C.

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<sup>1</sup> *E.coli* plasmid preparation by CsCl density gradient centrifugation.

### 2.1.2 *Chlamydomonas reinhardtii*

#### 2.1.2.1 Strains

*Chlamydomonas reinhardtii atpB* -deletion mutant CC-373 (*ac-u-c-2-21*), obtained from the *Chlamydomonas* Genetics Centre at Duke University, NC, USA, was used as the recipient for chloroplast transformation. The strain is light sensitive and non-photosynthetic due to a partly deleted *atpB* gene encoding the  $\beta$ -subunit of the chloroplast ATP synthase. The 2.5 kb deletion extends approximately half way into the 3' end of the *atpB* gene and adjacent inverted repeat (IR) (Blowers et al., 1989) (Fig. 6). In the transformed mutant the 3' *atpB* region and the IR region will be complemented by homologous recombination and the chimeric gene construct will be integrated into the chloroplast genome (Fig. 6). Transformants can be selected for restoring the photosynthetic capacity of the cells (Blowers et al., 1989).

+157, a *C. reinhardtii* chloroplast transformant, was used for comparison of RNA abundance with the +20-transformants tested in this work. The transformant contains an *rbcL*-GUS construct with an unmodified version of the 5' UTR.

MU7 (Salvador et al., 1993), another *C. reinhardtii* chloroplast transformant, was used in this work in order to be able to quantify the amount of GUS transcripts in +20-transformants. MU7 contains an *rbcL*:GUS construct with an unmodified version of the 5' UTR (*rbcL* sequence up to +97). The enhancer (+126), needed for maximum transcription, is not included in MU7.

#### 2.1.2.2 Growth of Algae

CC-373 –cultures were maintained in low-light conditions ( $\sim 0.05 \mu\text{mol}/\text{sec m}^2$ ) at room temperature (RT). The medium was liquid HSHA, made of high salt (HS)-medium (Sueoka, 1960), supplemented with potassium acetate (2,5 g/l).

Prior to transformation, mutant cells were grown on a shaker and supplied with fresh HSHA-medium daily to keep growth in log phase. Cells were plated on HSHA - agar and maintained in low light a few hours before transformation.

MU7, + 157 and photosynthetic transformants of CC-373 were grown at RT in HS-medium on agar plates or in HS-liquid cultures. High-density cultures were grown in glass tubes in a water bath at 32°C with continuous mixing by air with 2%

CO<sub>2</sub>. Transformants were grown in continuous light or in a 12 h light/12 h dark regime.

### **2.1.2.3 Chloroplast transformation**

Chloroplasts were transformed by the biolistic method as described previously (Blowers et al., 1989). Mutant CC-373 cells were bombarded with DNA coated 0.6 µm gold particles, using the helium-driven particle delivery system (PDS-1000/He; Bio-Rad) according to the Bio-Rad's biolistic protocol and bombardment parameters for algae at 1300 psi.

Stable transformants were selected by growth in high light on HS agar plates and grown in liquid HS-medium (section 2.1.2.2) for analyses of gene expression.

### **2.1.2.4 Isolation of nucleic acids**

Total genomic DNA was extracted as described (Blowers et al., 1989)

Total RNA was isolated at 11 hours in the dark and at one hour in the light from cultures grown in 12 h light/ 12 h dark cycles (Salvador et al., 1993).

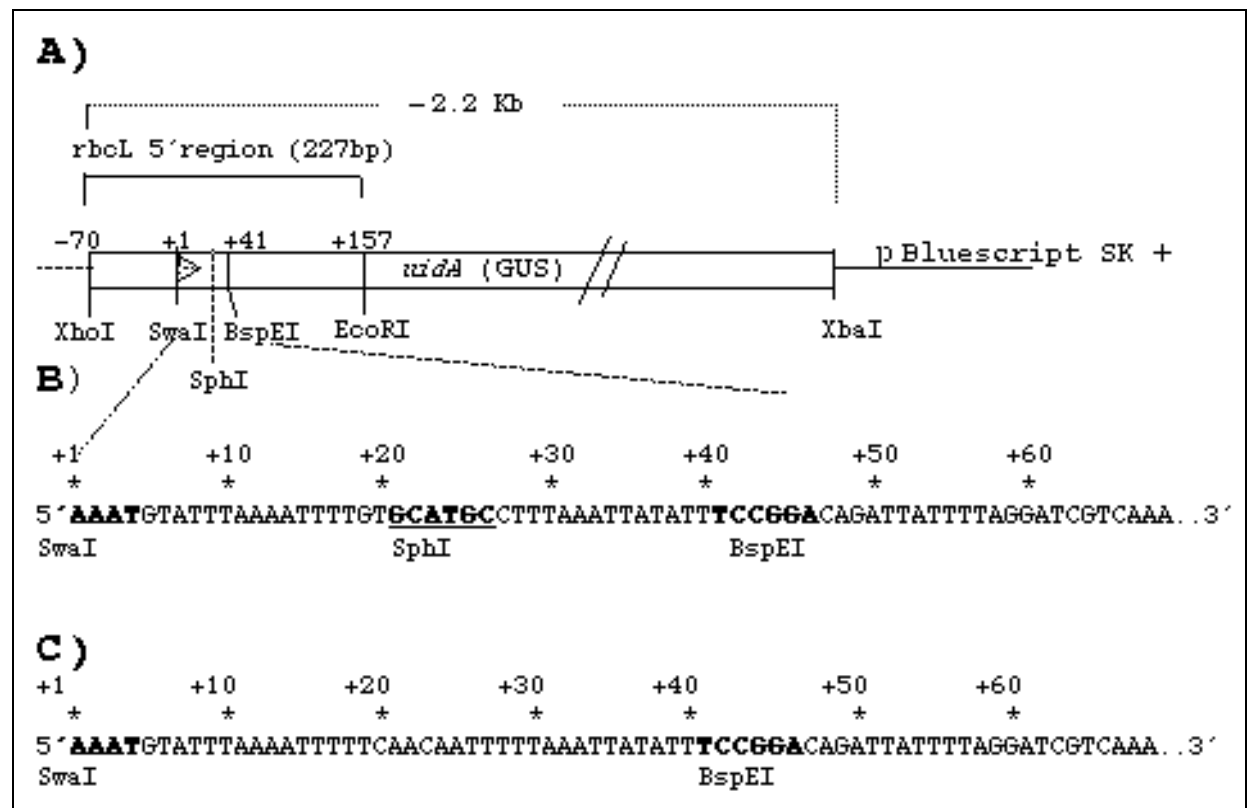
## **2.2 Plasmids**

### **2.2.1 +19/SK+ plasmid**

A ~5kb plasmid, p +19/SK+, containing the *rbcL* 5' region from position -70 to +157 relative to the start site of transcription at +1 fused 5' to the coding region of the GUS reporter gene, was used as the starting plasmid for all chimeric GUS constructs and for the generation of the final transformation vectors. The plasmid is identical to plasmid +157/SK (Anthonisen et al., 2001), with one exception: p+19/SK+ contains a *SphI* site (GCATGC) from position +19 to +27 (relative to transcription start + 1) in between the *SwaI* /*BspEI* sites of plasmid +157/SK+ (Fig. 5).

Plasmid +157/SK+ is based on pBluescript SK+ (Stratagene) and harbours a blunt-ended 227 bp DNA fragment from the 5' end of the *C. reinhardtii* chloroplast *rbcL* gene (extending from position -70 to position +157) cloned into the polylinker of the

pBluescript SK+. Downstream from the cloning site is the *E. coli uidA* ( $\beta$ -glucuronidase, GUS gene) (Fig.5).



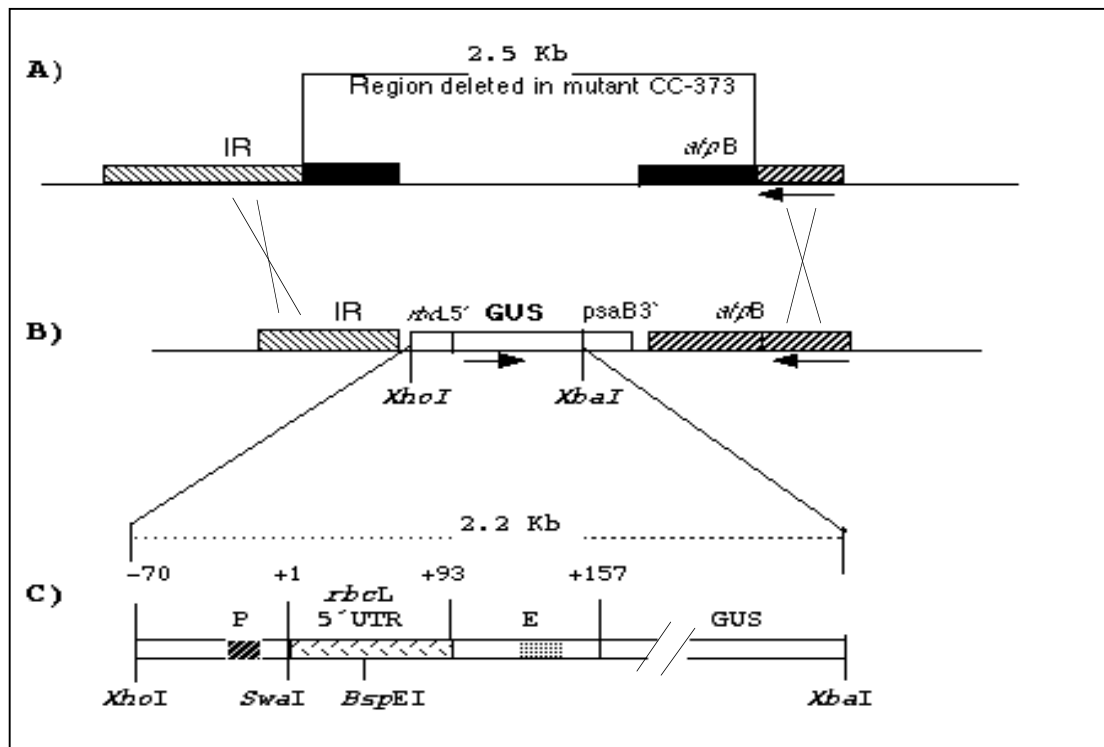
**Fig. 5 The plasmid +19/SK+ and restriction sites**

- A)** Schematic drawing of the 5' region of the *rbcL* gene cloned in front of the GUS -coding region in p+19/SK+. The 227 bp DNA sequence contains the *rbcL* promoter, its 5'UTR (positions +1 to +92), some of the *rbcL* coding sequence and a transcription enhancing sequence around position +126. XhoI/XbaI sites flank the *rbcL* 5': GUS chimera. The plasmid contains an ampicillin resistance gene for selection in transformed *E. coli* (not shown). Restriction sites used for cloning were SmaI and BspEI. The figure is not drawn to scale.
- B)** Nucleotide sequence at the *rbcL* 5' end in p+19/SK+. The SmaI, SphI and BspEI - sites are shown in bold type. The SphI site in p+19/SK+ in position +19 is lost upon cloning of oligonucleotides into the SmaI/BspEI digested +19/SK+ vector. Numbers above the sequence indicate the nucleotide positions relative to the start site of transcription at +1.
- C)** The original *rbcL* 5' end sequence without the SphI site.

### 2.2.2 +19/32 plasmid

The ~ 11 kb plasmid developed in our laboratory was used as the *C. reinhardtii* chloroplast transformation vector. It is based on plasmid pCrc32 (Salvador et al., 1993), a pUC – derived vector that contains an ampicillin–resistance gene as a selectable marker in *E. coli*. The +19/32 plasmid harbours a chimeric (*rbcL* [ *rbcL* sequences as in p+19/SK+]: GUS: *psaB* 3' end) construct flanked by *atpB* -IR

sequences complementing the chloroplast DNA deletions in the recipient mutant *Chlamydomonas* strain CC-373 (section 2.1.2.1) as explained in Fig. 6.



**Fig. 6** Schematic representation of the deletion in CC - 373, the transformation vector +19/ 32 with targeting regions and restriction sites in *p* +19/SK+ and *p* +19/32 used for cloning

**A)** The 2.5 kb deletion (black boxes) in the photosynthetic mutant strain CC-373.

**B)** The *Chlamydomonas* transformation vector *p* +19/32. *XhoI/XbaI*-restriction sites flank the chimeric *rbcL:GUS* construct. The *atpB* – *IR* (inverted repeat) sequences complement the deletions in the mutant CC-373. The two crosses designate the two homologous recombination events via the flanking *atpB-IR*-sequences, which promote stable incorporation of the foreign chimeric gene construct. Horizontal arrows indicate direction of transcription.

**C)** Unmodified *rbcL: GUS* chimera in *p*+19/SK+. Promoter (P) and enhancer (E) are indicated.

In the last cloning step all DNA constructs were inserted between *XhoI/XbaI* sites in *p*+19/32. The 2.6 kb reporter gene construct were now made of modified *rbcL* 5'UTR, *uidA* (*GUS*) and the *psaB* 3'UTR.

## 2.3 DNA Manipulation Techniques

### 2.3.1 Oligonucleotide annealing and phosphorylation

Complementary oligonucleotides were obtained from MWG Biotech AG, Ebersberg, Germany. The oligonucleotides were re-suspended in sterile distilled water at equal concentrations of 100 pmol/μl. Pairs of oligonucleotides (500 pmoles each) were annealed by incubating at 100°C for two minutes followed by slow cooling to room

temperature for 15-20 minutes (Sambrook and Russell, 2001).

Phosphorylation of the resulting double stranded DNA at the 5' ends was carried out by mixing T4-polynucleotide kinase (10 u/μl), polynucleotide kinase buffer (10 X) and ATP (10 mM) allowing incubation at 37°C for 1 hour.

### **2.3.2 Restriction endonucleases**

Restriction enzymes from New England Biolabs, were used according to the supplier's recommendations.

### **2.3.3 Agarose Gel Electrophoresis**

Agarose was used at 1% concentration in 1X TAE buffer (Sambrook and Russell, 2001). Bands were visualized by ethidium bromide staining (0,25 μg/ml).

Electrophoresis was performed in 1X TAE buffer for 30 to 60 minutes at 80 V. DNA-samples were mixed with gel loading buffer as described (one-tenth of total sample volume) before loading onto the gel (Sambrook and Russell, 2001). The 1kb plus ladder (Invitrogen) was used as a molecular weight marker to estimate the size of DNA fragments. 1.3 % agarose was used for isolating oligonucleotide fragments.

### **2.3.4 Isolation of DNA fragments**

Prior to cloning DNA fragments were separated on agarose gels. After electrophoresis a "well" was made in the gel below the target DNA fragment using a razor blade. A dialysis membrane was placed into the well in order to prohibit the DNA from migrating out of the well during electrophoresis. The well then contained the DNA fragment as a sharp band adjacent to the membrane and could be removed with a pipette. The DNA sample was further purified by phenol/chloroform extraction and ethanol precipitation.

### **2.3.5 Ligation**

T4 DNA ligase (New England Biolabs) was used as recommended by the protocol (Sambrook and Russell, 2001). In this work in a 10 μl ligation reaction optimum vector: insert ratio was approximately 1:5 in the first subcloning and 1:1.3 in the final cloning step.

## 2.4 DNA sequencing

Recombinant plasmids were sequenced by the Sanger dideoxy method (MWG-Biotech, Martinsried, Germany) to verify that the correct changes to the *rbcL* 5' UTR had been introduced using a GUS primer (5' - CGCGCTTCCCCACCAACGCTG – 3').

## 2.5 DNA/RNA quantification

The dot spot method was used estimate the concentrations of DNA-fragments isolated by gel electrophoresis and total DNA from *E.coli* transformants (Sambrook and Russel, 2001). More accurate spectrophotometric measurements at 260 nm were performed on samples from maxi-prep DNA and total DNA/RNA samples from *Chlamydomonas* (Sambrook and Russel, 2001) using an UV-visible spechtrphotometer (Bergman).

## 2.6 Hybridization analysis methods

### 2.6.1 Radioactive probes

Probes used for hybridization of DNA and RNA gel blots were labelled with [ $\alpha$  -  $^{32}$ P] dCTP, using the Klenow fragment of *E.coli* DNA polymerase I (Feinberg and Vogelstein, 1983). The half-life of radio-labeled nucleotides was approximately two weeks.

Zeta-Probe membranes from slot blot and northern blot were hybridized to the entire coding region of the radiolabeled GUS gene (~1.9 kb *Bam*HI-*Sac*I restriction fragment from plasmid pBI221; Clontech, CA, USA). In Southern blot the radiolabeled *atpB* -probe was a ~0,7kb *Hpa*I/*Eco*RV restriction fragment released from the vector pCrc*atpB* containing an internal portion of the *C.reinhardtii* chloroplast *atpB* gene (Blowers et al., 1990).

### 2.6.2 DNA slot-blot analysis

An initial screening of colonies for GUS content was carried out by the slot blot method as described previously (Blowers et al., 1990). Samples of genomic DNA (0.5



µg/slot) were denatured at 65°C in the presence of 0.3 M NaOH, applied to the assembled slot blot apparatus (PR600, Hoefer Scientific Instruments) and blotted onto a nylon membrane (ZetaProbe; BioRad). After transfer, DNA was covalently linked to the membrane by exposure to shortwave UV-light and the membrane was hybridized with the random primer-labeled GUS probe (section 2.6.1). Hybridization and wash steps were carried out by the BioRad ZetaProbe protocol (Church and Gilbert, 1984). Blots were carefully placed target side up in plastic wrap and exposed to X-ray film (Biomax MS, Kodak) at - 80°C.

The exposure time required depended on the specific activity of the labelled probe as well as the abundance of the target. After washing, the strength of the radioactive signal on the membrane was therefore measured using a Geiger counter. Typically exposure was carried out overnight with an intensifying screen (Kodak).

### **2.6.3 Southern blot**

1.5 µg genomic DNA was *HindIII* /*KpnI* digested and the resulting restriction fragment mixture was separated in a 1% agarose gel alongside a 1 kb Plus DNA Ladder (Invitrogen).

A picture was taken of the gel for size determinations of the hybridization bands later on. The DNA bands were further transferred to a ZetaProbe nylon membrane using the alkaline blotting and capillary pull procedure as described in the BioRad ZetaProbe manual. The random primer labeled *atpB* sequence (section 2.6.1) was used as the hybridization probe. UV- crosslinking, probing, washing and exposure to X-ray film was carried out by following the same protocols as in section 2.6.2.

### **2.6.4 Northern blot**

Total RNA (4 µg) was denatured with formamide/formaldehyde, size-separated by electrophoresis in a 1.3% agarose gel and blotted onto a Zeta-probe nylon membrane.

The membrane was hybridized to the random primer labeled GUS probe (section 2.6.1). UV- fixation, probing, washing and exposure to X-ray film, was carried out by following the same protocols as in section 2.6.2.

### **2.7 Secondary structure prediction**

The secondary structures of the 5' end of transcripts were predicted by the Burnet Institute Mfold server (<http://mfold.burnet.edu.au/>). The folding is based on calculation of the maximum free energy change (Zuker, 2003).

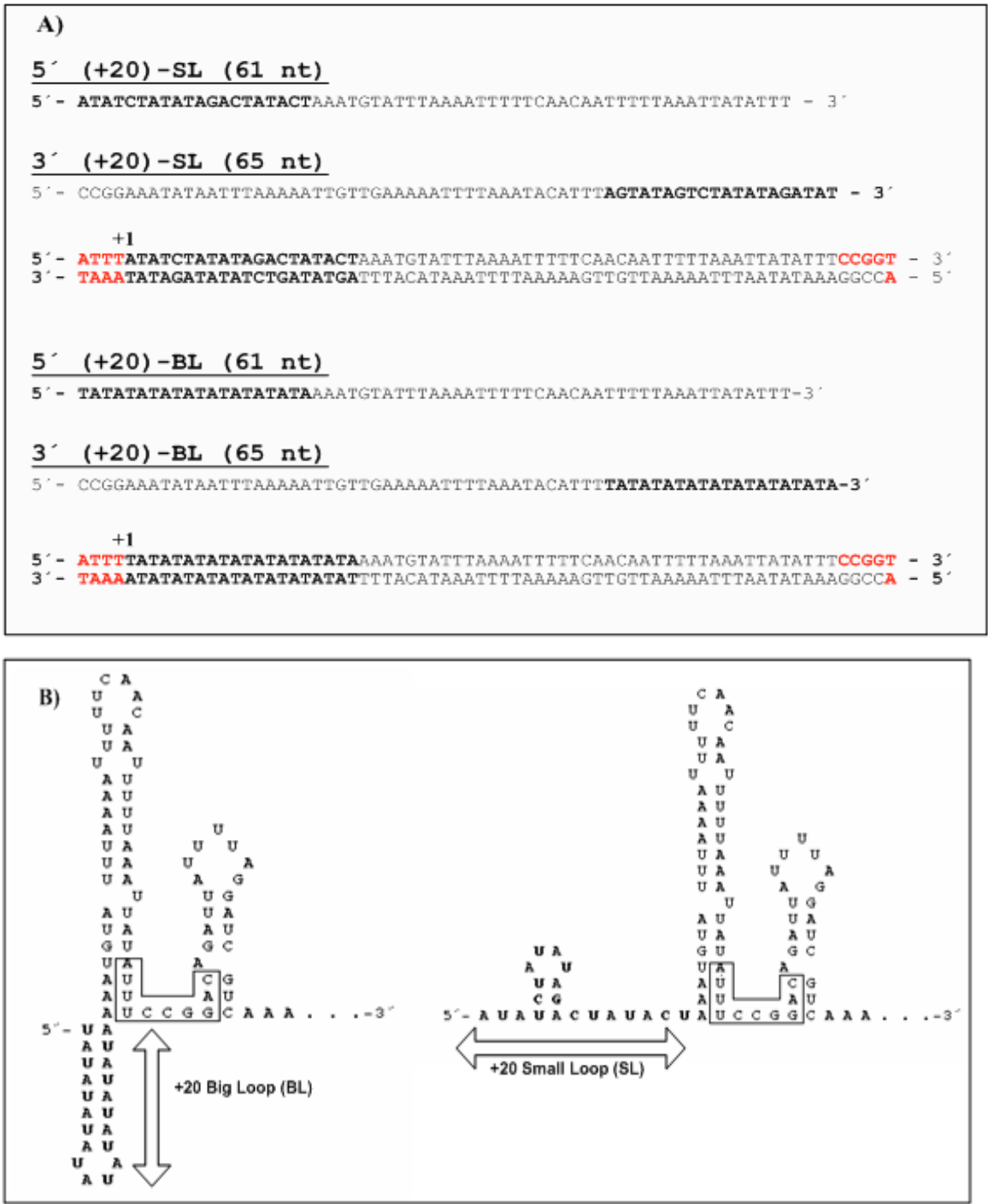
### 3 RESULTS

#### 3.1 Construction of plasmids p(+20) - SL and p(+20) – BL

##### 3.1.1 Subcloning; the (+20) - SL/Sk+ and (+20) - BL/Sk+ plasmids

Complementary oligonucleotides (Fig. 7A) containing the sequence between the *SwaI* and *BspEI* sites of the *rbcL* 5'-UTR (Fig. 5), plus the 20 extra nucleotides (nts), Big-Loop (BL) and Small-Loop (SL) (Fig. 7 A,B), added immediately downstream of the *rbcL*-gene transcription start site +1, were annealed to form blunt 5' ends and *BspEI*-compatible 3' ends (Fig. 7A). The sequence +1 to +41 was removed from the *rbcL* 5' region (Fig. 5) of plasmid +19/SK+ with *SwaI* (ATTT/AAAT) and *BspeI* (T/CCGGA), creating a blunt end and a 4 nts 5'-CCGG overhang, respectively (Fig. 7A). The large (~5kb) fragment of the vector was isolated from an agarose gel as described in section 2.3.4. The annealed oligonucleotides were ligated into the isolated ~5kb plasmids creating plasmids p(+20)-SL/Sk+ and p(+20)-BL/Sk+ (Fig. 7A).

To determine the transformation efficiency and testing the competency of the cells, a positive control transformation was performed with 1 ng of plasmid +19/SK+. Approximately 100 antibiotic-resistant transformants grew on the control LB-plates. Several colonies (five to ten) also grew on the plates with cells transformed with the (+20)-BL/Sk+ and (+20) -SL/Sk+ - ligations. Plasmids from overnight cultures of single colonies were digested and run on an agarose gel to confirm the size of the resulting fragments.



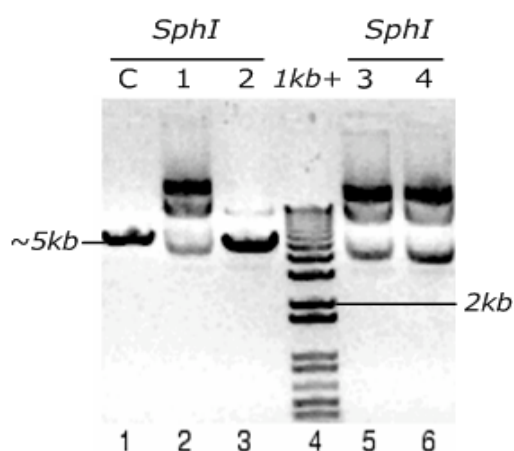
**Fig. 7 A) Oligonucleotides before and after annealing and their insertion into the *SwaI*/*BspEI* restricted +19/SK plasmid, creating a modified 5' end of the *rbcL* 5'UTR.**  
The start site of transcription is marked +1. The 20 base pairs are inserted between the *rbcL* 5'UTR and upstream promoter sequences.  
The plasmid compatible ends are shown in red and are not part of the ligated DNA insert.

***B) Predicted RNA secondary structures at the 5' end of transcripts of the (+20)-SL and (+20)-BL constructs.***

*The predicted secondary structures by the Burnet Institute MFOLD server (section 2.7) are re-drawn for uniform and space-saving appearance. Open boxes indicate a previously delineated sequence element important for transcript stability (Anthonisen et al., 2001).*

### 3.1.2 Test-digestions of (+ 20)-SL/Sk+ plasmids

Plasmids isolated from four randomly picked transformants were cut with *SphI* (Fig. 8); the p(+20)-SL/Sk+ plasmids were not expected to be cut by the enzyme, as the sequence between the *SwaI* /*BspEI* sites in the original +19/SK+ plasmid containing the *SphI* site was replaced by the sequence of the oligonucleotides (Fig. 5). Plasmids from transformants 1, 3 and 4 appeared to contain the SL-insert, as they were not cut by *SphI*. DNA from transformants 3 and 4 was further digested with *SwaI* (Fig. 9).



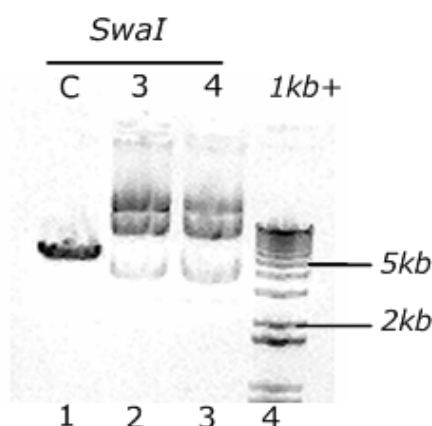
**Fig. 8 *SphI* digestion of plasmids isolated from +20-SL transformants.**

(Lanes are given below gel).

Lane 1: (C=control), +19/SK+ plasmids.

Lane 2,3,5 and 6: Plasmids from transformants 1,2,3 and 4.

Lane 4: The 1 kb plus ladder.



**Fig. 9 *SwaI* digestion of plasmids isolated from +20-SL transformants 3 and 4.**

(Lanes are given below gel).

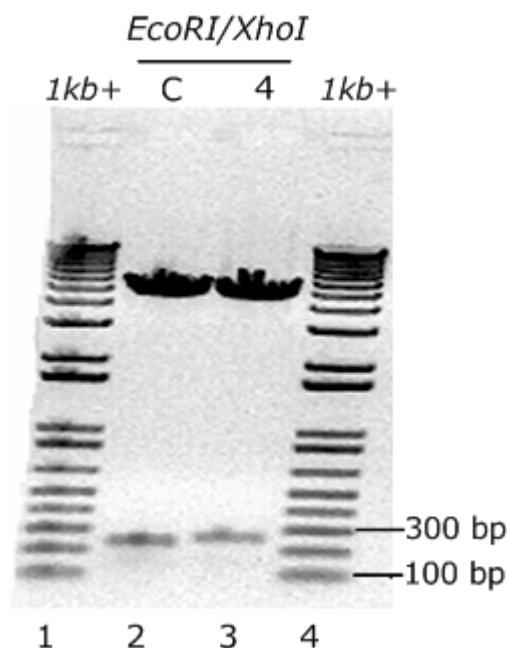
Lane 1: (C=control) +19/SK+ plasmids.

Lane 2 and 3: Plasmids from transformants 3 and 4.

Lane 4: The 1 kb plus ladder.

The original +19/SK+ plasmid was expected to be cut by this enzyme in a single position producing a ~5 kb linear fragment. However, DNA from correct transformants was not expected to be cut since cloning of the oligonucleotides into the *SwaI* site destroys the site (Fig.5). As predicted, plasmids from the two transformants were not cut by *SwaI*. Transformant 4 was chosen for subsequent cloning steps.

Plasmid DNA isolated from transformant 4 was cut with *EcoRI/XhoI* as a final control before sequencing (section 2.4) to verify that the correct sequence had been introduced (Fig. 10).



**Fig. 10 *EcoRI/XhoI* digestion of plasmid DNA isolated from transformant 4.**

(Lanes are given below gel).

Lane 1 and 4: The 1 kb plus ladder.

Lane 2: (C=control), p +19/SK+.

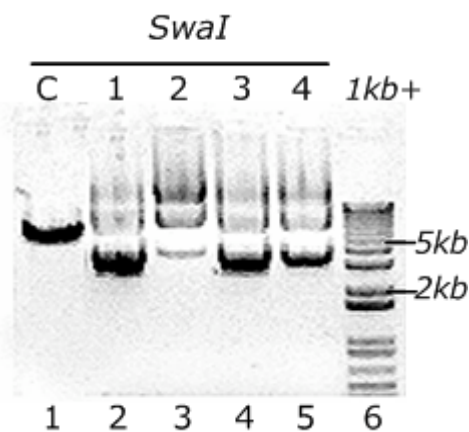
Lane 3: Maxi-prep-DNA from selected transformant cut with *EcoRI/XhoI*.

The control, the original +19/SK+ plasmid, was predicted to release a *EcoRI/XhoI*-fragment of ~230 bp whereas the plasmid from transformant 4 should release a fragment of ~250 bp; this was indeed observed, as shown in Fig. 10. By running two ladders on the same gel, the small difference in size between the fragments could be clearly demonstrated. Finally, sequencing was verified the incorporation of the SL-insert into the plasmid (data not shown).

### 3.1.3 Test-digestions of (+ 20)-BL/Sk+ - plasmids

To test whether isolated plasmids contained the correct + 20-BL-construct, the same strategy was used for the BL-insert as for the SL-insert. See previous section.

This time, plasmids were not checked by cutting with *SphI*, since *SwaI* and *EcoRI/XhoI* digestions were deemed to be sufficient to find the right construct (Fig. 11 and 12).

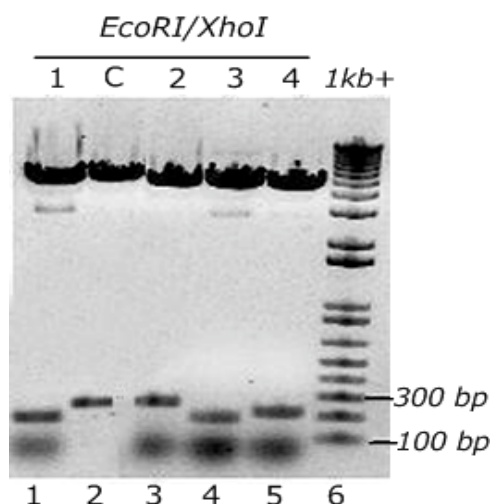


**Fig. 11 *SwaI* digestion of plasmids isolated from *E.coli* cells transformed with the +20-BL/Sk+ construct** (Lanes are given below gel).

Lane 1: (C=control) p +19/SK+.

Lane 2-5: Plasmids isolated from transformant bacteria. Plasmids isolated from transformant 2 (above gel) seemed to be different in size than plasmids from transformants 1, 3 and 4.

Lane 6: 1 kb plus ladder.



**Fig. 12 *EcoRI/XhoI* digestion of plasmids isolated from *E.coli* cells transformed with the +20-BL/Sk+ construct.** (Lanes are given below gel).

(Lanes are given below gel).

Lane 2: (C=control) p+19/SK+ from maxiprep.

Lane 1, 3, 4 and 5: Plasmids isolated from transformants.

Lane 6: The 1 kb plus ladder.

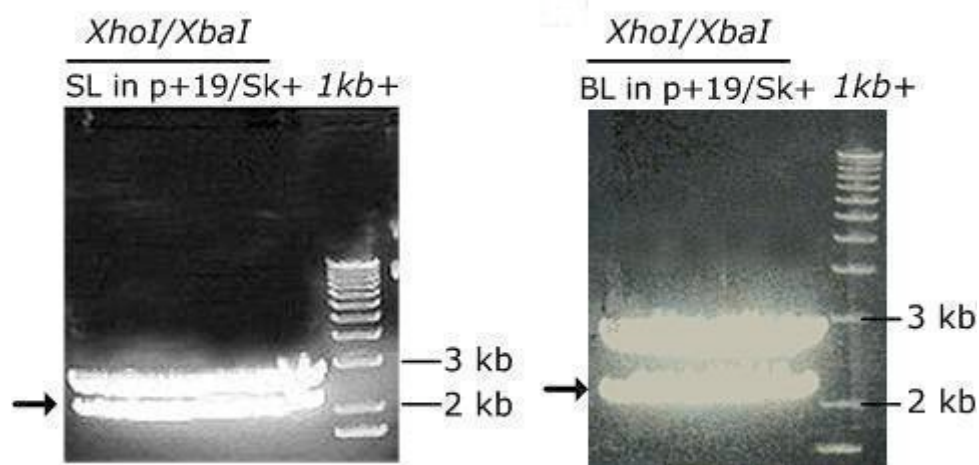
None of the plasmids isolated from transformants 1- 4 was cut by *SwaI* (Fig. 11). However, the results from the *EcoRI/XhoI* digestion, showed that only transformant 2 harboured the construct with the correct insert size (Fig. 12, lane 3). Plasmids from transformant 2 cut by *EcoRI/XhoI* produced a ~ 250 bp fragment, slightly bigger than the ~230 bp fragment produced by the control plasmid. The other plasmids produced fragments around 200 bp.

Next, a plasmid maxiprep was made from transformant #2 and DNA was quantified spectrophotometrically (section 2.5). Finally, the sequencing results verified the incorporation of the BL- insert into the plasmid (not shown).

### 3.1.4 Cloning (+20)-SL and (+20)-BL into the chloroplast transformation vector

The 5'UTR: GUS cassette including the additional 20 bp, was released from plasmids (+20)-SL/Sk+ and (+20)-BL/Sk+ with *XhoI* and *XbaI*, producing 2.2 kb and 2.8 kb fragments as expected (Fig. 13). The 2.2 kb fragments containing the GUS construct

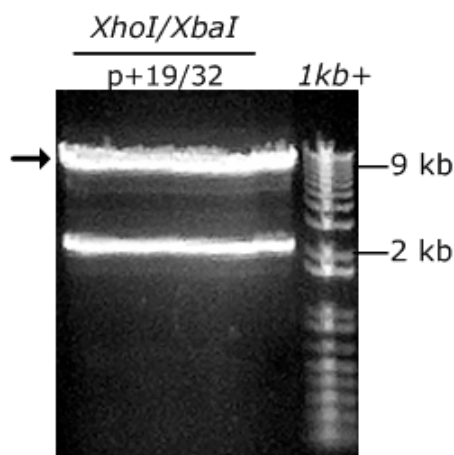
were isolated from the gel as described in section 2.3.4. The dot spot method was used to determine DNA concentrations.



**Fig. 13 Isolation of the *XhoI/XbaI*- SL-and BL-inserts from (+20)-SL/Sk+ and (+20)-BL/Sk+, respectively.** The photos show the agarose gels prior to isolation of the 2.2 kb *XhoI/XbaI* fragments that contain the +20-SL and +20-BL - GUS constructs. The arrows indicate the 2.2 kb fragment containing the GUS construct.

To construct the final plasmids p (+20)- SL and p (+20) - BL, the *rbcL*: GUS fragments released by *XhoI/XbaI* digestion were cloned into the *Chlamydomonas* transformation vector +19/32, cut with the same enzymes (Fig. 14).

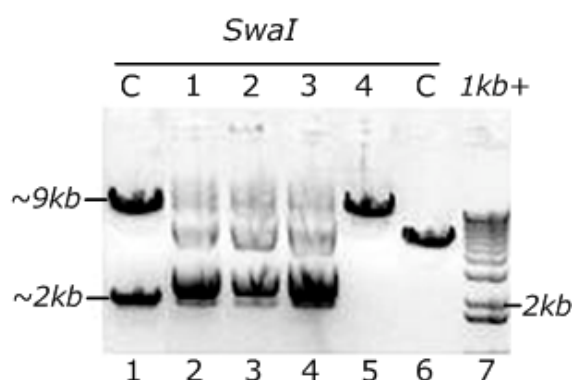
The large ~9 kb fragment produced upon digestion of p+19/32 with *XhoI/XbaI* (Fig. 14) was isolated, quantified as before, and used in the final ligations with the 2.2 kb inserts. Transformants were grown on LB agar and plasmids were isolated from transformants as described previously.



**Fig. 14 The transformation vector cut with *XhoI/XbaI* and separation of the two resulting fragments by gel electrophoresis.** The ~ 9 kb fragment (arrow) representing the vector without the GUS construct was isolated.

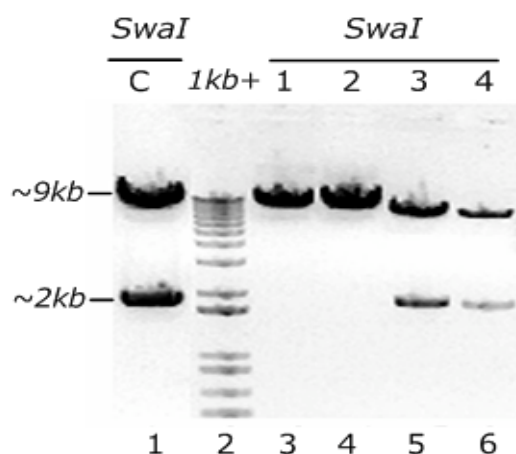


Miniprep DNA was cut with *SwaI* (Fig. 15 and Fig. 16). The original p +19/32 transformation vector contained two *SwaI* sites, one at position +1 in the *rbcL* 5' region (Fig. 5) and one outside the *XhoI/XbaI* fragment in the *Chlamydomonas* sequence (not shown). *SwaI* digestion of this plasmid therefore resulted in two fragments. However, correct recombinant plasmids were expected to be cut in a single position by *SwaI*, since the *SwaI* restriction site at +1 was destroyed as described in section 3.1.2. In fact, the transformants obtained from transformant 4 (Fig. 15) and from transformant 1 and 2 (Fig. 16.) carried the desired plasmids and, therefore tested positive for the SL-insert and BL –insert, respectively.



**Fig. 15 Test digestion of p(+20)-SL, with *SwaI*.**

(Lanes are given below gel)  
Lane 1: (C=control) p +19/32.  
Lane 2-5: Transformants 1 - 4.  
Lane 6: (C=control) p +19/SK+.  
Lane 7: The 1 kb plus ladder.



**Fig. 16 Test digestion of p (+20)-BL, with *SwaI*.**

(Lanes are given below gel).  
Lane 1: (C=control) p +19/32.  
Lane 2: The 1 kb plus ladder.  
Lane 3-6: Transformants 1 - 4.

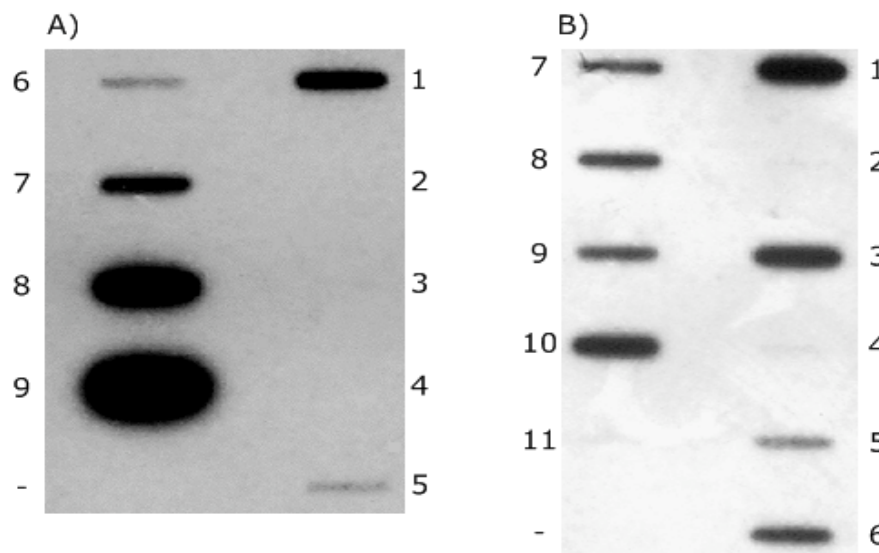
Plasmids isolated from transformants 3 and 4 (Fig. 16) produced bands of approximately 2 and 9 kb, identical to the control, suggesting that these transformants contained the original transformation vector +19/32. This implied that the 9 kb band isolated from the gel in Fig. 14 also contained linearised plasmid i.e singly cut plasmids. Since *E.coli* is not readily transformed by linear pieces of DNA (Smith et al., 1981) the plasmids must have religated upon transformation allowing replication in bacterial host cells.

Plasmids were isolated (by the maxiprep method) from the p(+20)- SL transformant # 4 and p(+20)-BL transformant #1 and sequenced before transformation into *Chlamydomonas*. Sequencing confirmed the insertion of *rbcL* 5'UTR: GUS constructs containing the +20 extensions to the 5' end into the *Chlamydomonas* transformation vector.

### 3.2 Screening for transformants harbouring the foreign GUS gene

Chimeric (+ 20) - SL and (+20) - BL plasmids (isolated by the maxiprep method) were used to biolistically transform chloroplasts of *C. reinhardtii* cells. A number of transformants were obtained. Selected transformants were subcultured in order to test progeny for photoautotrophic growth. Total DNA was isolated from 9 p(+20)- SL transformants and 11 p(+20)-BL-transformants (see section 2.1.2.4). The presence of the foreign chimeric [*rbcL* 5'UTR: GUS: *psaB* 3' region] constructs in the chloroplast genome was verified by slot-blot analyses using the radiolabeled coding region of the GUS gene as a probe (Fig. 17).

Due to the selection process on HS – medium (without acetate) and high-light conditions, all surviving algae must have incorporated the *atpB* gene complementing the cpDNA deletion (Fig. 6). Reconstitution of the *atpB* gene results in light-tolerant (i.e photosynthetic) cells and therefore the phenotype of all transformants would be the same irrespective of the incorporation of the chimeric GUS gene into the chloroplast genome. Since transformants sometimes incorporate the *atpB* sequences without embedding the *rbcL*: GUS chimeric gene, it was necessary to screen for GUS in any given transformant obtained from independent isolates. The slot blot analysis showed that transformants p(+20)-SL 8 and 9 and transformants p(+20)-BL 3 and 1 contained most copies of the GUS gene. These were chosen for further analysis of homoplasmy.



**Fig. 17** Autoradiogram of slot blots probed with radiolabeled *GUS* DNA.

Total cellular DNA (0,5 µg) from transformants was immobilized on a Zeta-Probe membrane. The membranes were hybridized over night at 65 °C to the random primer labelled *GUS* probe. Membranes were washed and exposed to X-ray film at -80 °C for 6 h to 12 h. By comparing the signal strengths, the relative *GUS* content could be estimated in each sample. **A)** DNA from 9 different p(+20)-SL transformants, and **B)** from 11 different p(+20)-BL transformants.

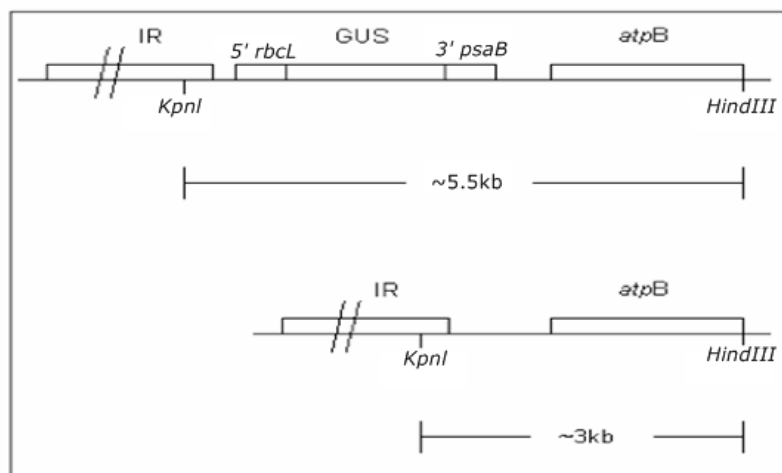
### 3.3 Homoplasmicity of transformants

As mentioned previously, the chloroplast of *Chlamydomonas* contains 50 to 80 copies of the ~200-kb circular DNA chromosome in one cell and DNA insertion during transformation can occur in more than one of these copies. A cell line that has transformed the chimeric *GUS* construct into all of the chromosomal copies is called a homoplasmic cell line.

DNA from photoautotrophically grown p(+20)-SL 8 and 9 and p(+20)-BL 3 and 1 showing the strongest *uidA* (*GUS*) signal were tested by Southern gel blot analysis for homoplasmicity. Total cellular genomic DNA was *HindIII* /*KpnI* digested, fractionated on an agarose gel and blotted onto a nylon membrane, before hybridization with the *atpB* probe (section 2.6.3).

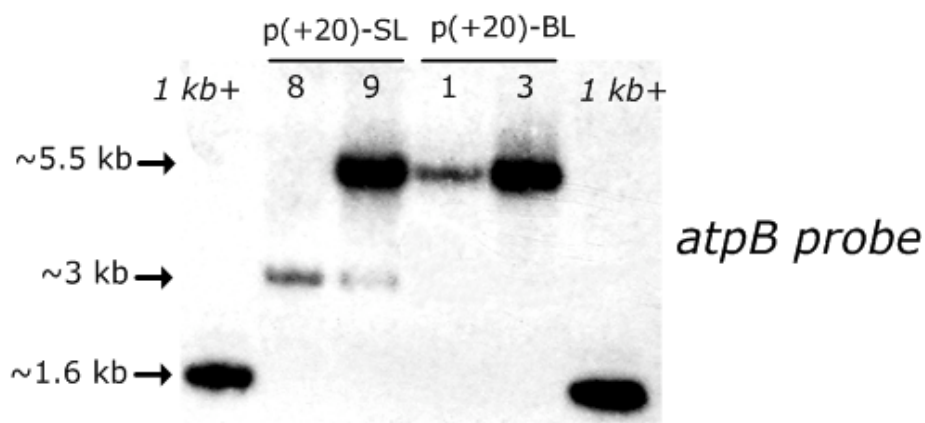
*atpB*- hybridizing restriction fragments were either ~5.5 kb or ~3 kb in size, depending on incorporation of the *GUS* gene (Fig. 18). The degree of homoplasmicity was indicated by the ratio of the two DNA fragments isolated from chloroplast transformants. As shown in Fig. 19, both the p(+20)-BL 1 and 3 transformants showed a homoplasmicity of 100% and seemed harbour the complete construct (~ 5.5 kb) of the reporter gene chimera in all chloroplast genomes. The p(+20)-SL 9 transformant showed a homoplasmicity of 80 - 90% and transformant p(+20)-SL 8

showed a homoplasmy of 0%. Transformants (+20)-BL 1 and (+20)-SL 9 were chosen for further analysis.



**Fig. 18 *atpB* containing restriction fragments in genomic DNA of *Chlamydomonas* transformants.** The *atpB* containing *KpnI*/*HindIII* fragments produce a ~3 kb fragment when cutting the DNA without the *GUS* containing reporter gene. When the reporter gene is present, a ~5.5 kb fragment is produced.

In order to obtain accurate results for subsequent analysis of transcript stability, only the cell lines being 80 (90) - 100% homoplasmic (with respect to the chimeric construct) were subjected to further analysis (Northern, RNA).



**Fig. 19 Autoradiogram of the Southern blot (+20-SL and +20-BL)**

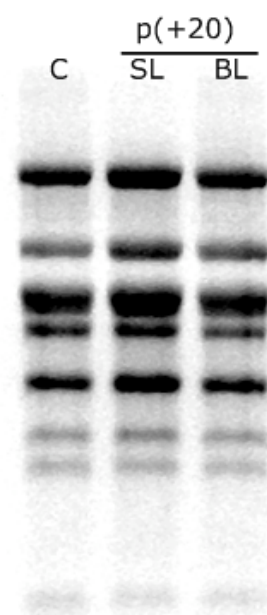
Autoradiogram of the Zeta-probe membrane shows the degree of homoplasmy of the *Chlamydomonas* transformants (+20)-SL and (+20)-BL. 1.6  $\mu$ g of total genomic DNA from transformants were cut with *HindIII* and *KpnI*, separated on a 1% agarose gel and, probed with the *atpB* probe after blotting to the membrane. X-ray film; 12- hours exposure at  $-80^{\circ}\text{C}$ .

The 1.6 kb fragment of the 1kb+ ladder hybridizes strongly to the *atpB* probe. Lanes from the left to the right; The 1kb+ DNA ladder, transformants p(+20)-SL-8 and 9, p(+20)-BL-1 and 3 and the 1kb+ DNA ladder. Sizes of fragments are shown to the left.

### 3.4 Accumulation of (+20) chimeric *rbcL*: GUS transcripts

Northern blot hybridization was used to determine whether the introduced changes affected the stability of GUS transcripts. In light of previous results (section 1.4), GUS transcripts were not expected to be stable. Total RNA was isolated from dark-adapted cells (section 2.1.2.4), denatured and separated on 1.3% agarose gels.

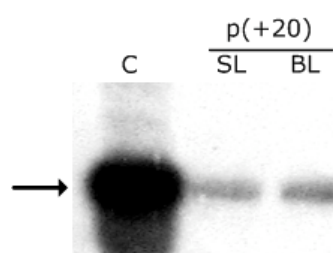
rRNA (Fig. 20), which makes up the bulk pool of RNA, was used to verify that the amount of total RNA loaded on the gel was approximately equal for each sample (Fig. 20).



**Fig. 20 Northern gel with RNA from *Chlamydomonas chloroplast* transformants *p* (+20)- SL and *p* (+20) - BL.**

Photograph of the EtBr stained agarose gel.

Total RNA from dark grown transformants was denatured and separated in a 1,3 % agarose gel. RNA amounts loaded per lane were 4 µg. (C=control), plasmid +157 containing an *rbcL*: GUS construct with the unmodified *rbcL* 5'UTR.



**Fig. 21 Autoradiogram showing accumulation of chimeric GUS transcripts in *Chlamydomonas chloroplast* transformants *p*(+20 )-SL and (+20)- BL .**

RNA was isolated from dark grown samples and transferred to a Zeta-Probe membrane by capillary blotting.

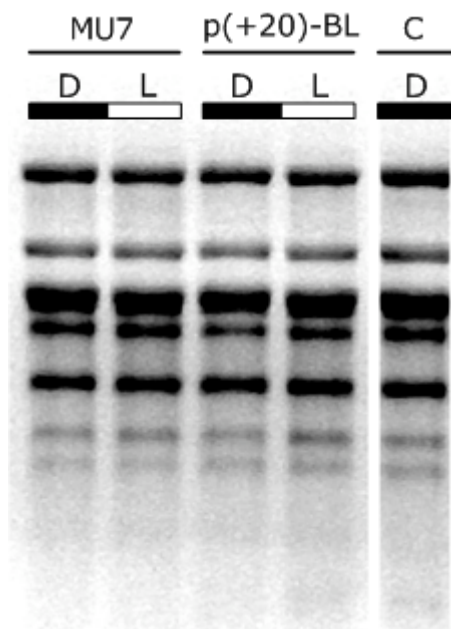
The Zeta-Probe membrane was hybridized to the random primer labelled GUS probe specific for the GUS gene. Hybridization bands were visualized after over night exposure to an X-ray film (14- hour exposure at  $-80^{\circ}\text{C}$ ). A strong

radioactive signal was shown for the +157 control ( $c$  = control). GUS transcripts are detectable in +20 transformants albeit at low levels.

The results from Northern hybridization showed that of the + 20 chimeric constructs, both SL and BL transcripts, accumulated in the transformants, although to much lower levels than in the control transformant +157 (Fig. 21). In order to be able to quantify the amount of GUS transcripts in +20 transformants, the samples were run on a second RNA gel (section 3.4.1) with RNA from transformant MU7 as a control. MU7 accumulates approximately 10 times lower levels of the GUS transcripts than the +157 transformant due to the lack of a transcriptional enhancer (section 2.1.2.1).

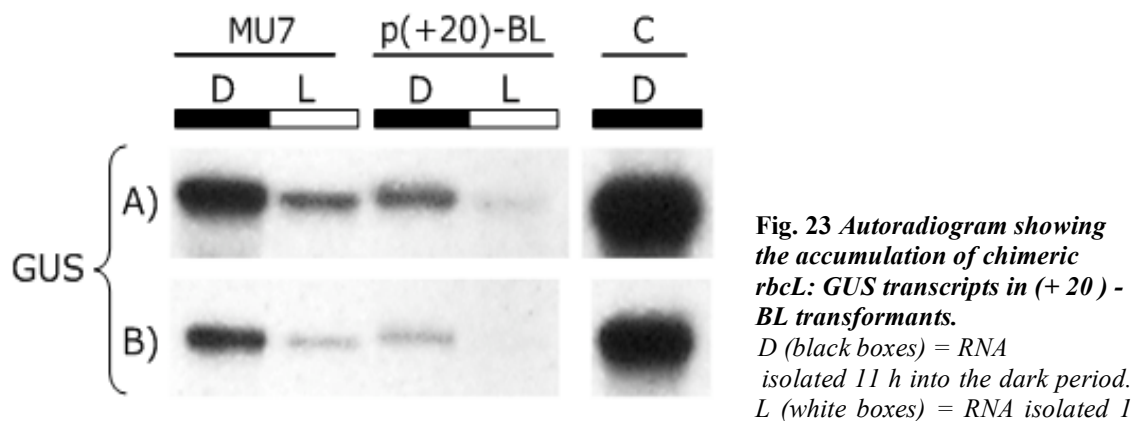
### 3.4.1 Semi-quantitative estimation of the stability of (+ 20) chimeric *rbcL*: GUS transcripts

RNA was isolated from cells grown in 12 h light/12 h dark periods at 1 h into the light period and 11 h into the dark period. Transformant (+20)-BL was chosen for this analysis because it was 100 % homoplasmic. In addition, a dark sample from the p+157 transformant was included. The gel in Fig. 22, illustrates that the amount of RNA loaded on the gel varies in an acceptable range.



**Fig. 22 Northern gel of RNA from the (+ 20) BL transformant.**

Photograph of EtBr stained agarose gel. Transformants from left to the right: Dark-isolated (D) and light-isolated (L) RNA samples of MU7, (+ 20) - BL and +157(c=control). All samples were run on the same gel. D (black boxes) = RNA isolated 11 h into the dark period. L (white boxes) = RNA isolated after 1 h in light.



h into the light period. The Zeta -Probe membrane was hybridized to the random primer labelled GUS probe specific for the GUS gene.

Hybridization bands were visualized by over night exposure to an X-ray film:

**A)** Membrane exposed to X-ray film for 16 h,  $-80^{\circ}\text{C}$ .

**B)** Membrane exposed to X-ray film for 7 h,  $-80^{\circ}\text{C}$ .

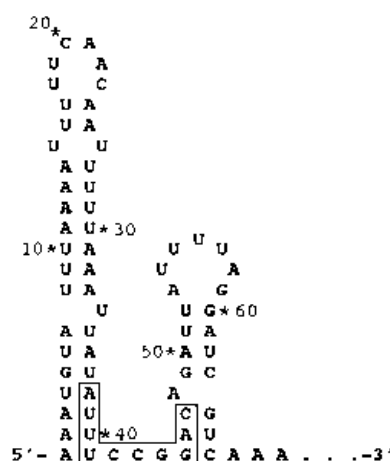
Transformants from left to the right: Dark-isolated (D) and light-isolated (L) RNA samples of MU7, (+ 20) - BL and + 157+(c=control). All samples were from the same autoradiogram.

The autoradiogram from the northern blot analysis (fig.23 A, B) revealed a strong signal from the dark-isolated MU7 sample, indicating that the GUS transcript accumulated to a relatively high level in this transformant ( $\sim 10\%$  of the RNA from the dark-isolated +157 transformant, which was expected since the enhancer was not included in MU7). For the light-isolated MU7 sample, a signal approximately 10 % of that of the dark sample is visible. The signal from the BL- dark-sample was approximately the same as the signal from the MU7- light sample. The signal from BL light sample was very weak and was significantly lower than the signal from the BL dark-sample, showing almost no accumulation of GUS transcripts in illuminated cells. The fact that (+20)-BL-transcripts accumulated (at least in the dark) was consistent with the results in the first northern analysis where both BL and SL – transcripts accumulated (Fig. 21). The GUS signal from dark-grown BL-transformants was comparable to the GUS signal from the MU7 light-grown samples and therefore the level of GUS transcripts in dark-adapted BL-transformants appeared to be  $\approx 1\%$  of the level in control transformant +157.

## 4 DISCUSSION

In this project, two reporter gene constructs (p+20-BL and p+20-SL) with modifications in the 5'UTR of the *Chlamydomonas* chloroplast *rbcL* gene were introduced into the chloroplast genome of *Chlamydomonas* to determine the effect of the 5'UTR modifications on transcript stability *in vivo*. The purpose was to assess to what extent nucleotides could be added to the 5' terminus of the *rbcL* 5'UTR chimeric reporter gene transcripts, before transcripts became unstable and susceptible to rapid degradation in *Chlamydomonas* chloroplasts.

The 5'UTRs of several chloroplast mRNAs have been shown to contain *cis*-acting elements that are required for stability of the transcripts (Salvador et al., 1993; Nickelsen et al., 1994; Eibl et al., 1999; Nickelsen et al., 1999; Singh et al., 2001; Salvador et al., 2004). In *Chlamydomonas*, a 10 nt *cis*-acting stability element required for longevity of *rbcL* transcripts is made up of nucleotides +38 to +47 in the 5'UTR, relative to the start site of transcription at +1 (Fig. 24). This element lies within a previously predicted secondary structure consisting of two stem-loops that are separated by a short single-stranded region (Anthonisen et al., 2001). The function of this *cis*-acting element (such as a possible binding site for a protective *trans*-acting factor(s) (section 1.2.3.3), depends on its accurate primary sequence in the single stranded region (+42 to +44) and secondary structure and/or sequence of other nucleotides (+38 to +41 and +45 to +47) (Anthonisen et al., 2001; Suay et al., 2005).



**Fig. 24** The predicted RNA secondary structure of nucleotides +1 to +69 of the *C. reinhardtii* *rbcL* 5'UTR (Anthonisen et al., 2001).

The 10 nts stability element (+38 to 47) is boxed.



#### 4.1 Effect on adding nucleotides to the 5' terminus of the *rbcL* 5'UTR

The results presented here suggest that transcripts of *rbcL*: GUS genes, with an addition of 20 nucleotides are quite unstable. The BL construct resulted in reporter-gene transcript accumulation  $\approx 1\%$  of the control, the unmodified +157 transcripts (Fig. 23). However, the transcripts still accumulated to levels that were detectable by RNA gel (Northern) blot analysis.

Previous studies, where nucleotides (nts) have been added to the *rbcL* 5'UTR 5' terminus, have shown that changes in the RNA conformation around the base of the stem of the *rbcL* 5' terminal stem-loop structure (+1 to +41) shown in fig. 24, resulted in abolished accumulation of chimeric (*uidA*) GUS transcripts (Salvador et al., 2004; Suay et al., 2005). Therefore, the constructs tested in this work were designed so that the structure and sequence of the original *rbcL* 5' region was maintained. Previous work has also shown that adding 6 unpaired nts (5'- AAGAAC -3') to the transcript's 5'-terminus did not change the stability of (*uidA*) GUS transcripts (Fig. 25, construct 1), whereas an addition of 10 nts (5'- AUAUAUAUAU -3') completely destabilized transcripts (Fig. 25, construct 2). The constructs tested in this project, containing the 20 nts extensions with predicted small and large stem-loop structures, are shown in fig. 25, construct 3 and 4. Altogether the results show that the addition of more than 6 nts destabilizes the chimeric (*uidA*) GUS transcripts.

It is tempting to suggest that the stem-loop structures of the +20 constructs could positively affect the stability of transcripts, since +10 transcripts, without a stem-loop structure, did not accumulate to detectable levels in northern analysis as did +20 transcripts (fig. 25). Yet it is not possible to make any conclusions, on a role of the BL and SL stem-loops in stability. Further investigations should add 20 nts that do not fold into a secondary structure.

Furthermore, it is also possible that specific ribonucleotides within the added RNA sequences (Fig. 25, shown in red) are recognized by *trans*-acting factors that are involved in stability and/or degradation, thus affecting the accumulation of the reporter gene transcripts.



## 4.2 Light/dark regulation

It has previously been observed that chimeric reporter gene transcripts containing only the 5'UTR part of the *Chlamydomonas rbcL* gene are substantially less stable in the light than they are in the dark (section 1.2.3.1)(Salvador et al., 1993). It has also been reported that altering the 10 nts *cis*-element in fig.24, 0 does not affect this difference in stability. Singh et al., (2001) found that another RNA sequence element between positions + 27 and + 41 in the stem-loop formed by the first 41 nts of the 5'UTR (Fig. 24), was the target for light-dependent RNA degradation of *rbcL* 5' UTR containing reporter gene transcripts *in vivo*. For the endogenous *rbcL* gene, a stem-loop structure in the coding region situated around position + 329 to +334 has also been described, whose loop portion around + 327 suppresses the destabilizing effect of the light-induced mRNA decay by the structured segment + 27 to +41. This stabilization might be through a direct or indirect (via proteins) physical interaction between the predicted stem-loops, in such a way that a postulated nuclease target responsible for the light-accelerated degradation is distorted.

In summary, these data suggest that there are two pathways of RNA breakdown in the chloroplast of *Chlamydomonas*, one light/dark regulated pathway shown to be linked to the redox state in the chloroplast (Salvador and Klein, 1999) and one light-independent pathway, involving the general *cis*-acting stability element (Fig. 24).

The MU7 transformant demonstrates that inclusion of the *rbcL* 5'UTR, containing the light-induced target in a reporter gene, renders its transcripts unstable in the light relative to the dark. In MU7 the first 97 nts of the *rbcL* gene are included, but not the stabilizing loop-structure in the *rbcL* coding region (+327). The results presented here show a light/ dark pattern of transcript levels for MU7, which is in agreement with earlier reports (Salvador et al., 1993). Only about 10 % of the transcripts survived after cells were exposed to light for 1 hour, implying that the half-life of the transcripts was drastically reduced in these cells. In the (+ 20) - BL transformant the levels of GUS transcripts decreased upon 1 hour of illumination (fig. 23). This decrease in light was also expected, since the sequences in the *rbcL* coding region were only included up to position + 157 (Fig.5).

### 4.3 +20-reporter gene transcripts are processed at their 5'ends

The mechanisms of RNA degradation in chloroplasts are not fully understood to date, and the components of the molecular machinery (exo- and endoribonucleases) that degrade mRNA are not known (section 1.2.4). A primer extension study performed on the +20-BL and +20-SL transcripts showed that +20-SL and BL transcripts were a target for a ribonuclease, because they were not 20 nts longer than the control (+157) transcripts (Uwe Klein, personal communication). Interestingly, the BL and SL were processed differently, possibly by a 5' → 3' exonuclease or an RNase E-type endoribonuclease (section 1.2.4). The exact role of this processing remains to be elucidated.

In future work, it would be interesting to isolate the putative *trans*-acting RNA binding protein(s) that are supposed to bind to the *cis*-acting 10-nts *rbcL* 5'UTR element (fig. 24) to protect the transcripts from rapid degradation. Furthermore, characterization of other factor(s) that might be involved in *rbcL* transcript stability is important in order to understand the molecular mechanisms underlying chloroplast mRNA accumulation.

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